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Evaluation of the antibacterial and wound healing activity of the crude and solvent fractions of leaves of *Acanthus polystachus* Delile

By

WUBANTE DEMILEW (B.Pharm, MPH)

A thesis submitted to the department of pharmacology, school of pharmacy, college of medicine & health sciences, University of Gondar in Partial Fulfillment of the Requirements for the degree of Master of Science in Pharmacology

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Wubante Demilew

Advisors: Seyfe Asrade (B.Pharm, MSc pharmacology)

Getnet Mequanint (B.Pharm, MSc pharmacology)

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This is to certify that the thesis prepared by Wubante Demilew entitled "Evaluation of antibacterial and wound healing activity of the crude and solvent fractions of leaves of *Acanthus polystachus* Delile (Acantheceae)" and submitted in partial fulfillment of the requirements for the degree of Master of Science in pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Name	Signature	Date
External Examiner_____	_____	_____
Internal Examiner _____	_____	_____
Advisor Seyfe Asrade (MSc)	_____	_____
Co-advisor Getnet Mequanint (MSc)	_____	_____

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List of Abbreviations and Acronyms

ANOVA	Analysis of Variance
BP	British Pharmacopoeia
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standard Institute
DNA	Deoxy Nucleic Acid
ECM	Extracellular Matrix
LPO	Lipid Per Oxidation
MBC	Minimum Bactericidal Concentrations
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
MMP	Matrix Metallo Proteinases
OECD	Organization for Economic Cooperation and Development
PDGF	Platelet Derived Growth Factor
RNA	Ribo Nucleic Acid
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SPSS	Statistical Package for the Social Sciences
TNF	Tumor Necrosis Factor
WHO	World Health Organization

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Abstract

Background: *Medicinal plants play indispensible roles to treat various* ailments*. Acanthus polystachus* ("Kosheshile" in Amharic) *is one of the medicinal plants used traditionally for treatment of wound and bacterial infections. However, to date, no scientific report could be found concerning the wound healing & antibacterial activities. Thus, the present study provides a scientific evaluation for the antibacterial and wound healing potential of the crude and solvent fractions of Acanthus polystachus leaves.*

Methods: *The crude extraction was carried out using 80% methanol and fractionated by chloroform, ethyl acetate followed by distilled water in increasing polarity. The antibacterial activity was evaluated using agar well diffusion &broth dilution techniques. The crude extract was prepared in 5% (w/w) and 10% (w/w) ointment & evaluated for wound healing activity using excision, infected & incision model in mice. The data was analyzed using Statistical Package for Social Sciences (SPSS version 20). One way analysis of variance (ANOVA) followed by Tukey test was employed & P-value <0.05 considered statistically significant.*

Results*: The crude extract, ethyl acetate & aqueous fractions showed significant dose dependent antibacterial activities against against S. aureus, S. pyogen and P. aeruginosa at 250 & 500mg/ml concentrations (P <0.001). The most susceptible bacterium to crude extract was S. aureus with mean zone of inhibition 16.33 & 15.00 mm at 500 & 250mg/ml respectively. Similarly, in ethyl acetate & aqueous fractions S. pyogen & P. aeruginosa were more susceptible (at 500 mg/ml) with mean zone of inhibition 14 & 11.67 mm respectively. Furthermore, only the crude extract & chloroform fraction revealed antibacterial activity against K .pneumonia. However, E. coli was not susceptible to the crude extract & all solvent fractions. Both 5% & 10% (w/w) ointments exhibited significantly reduced period of epithelialization, increased wound contraction rate & tensile strength compared to the negative control group (P<0.05). Better wound healing activity was observed in 10% (w/w) than 5% (w/w) & nitrofurazone ointment treated groups however the difference was failed to reach statistical significance.*

Conclusion: *These results collectively demonstrate that the crude extract & solvent fractions of A. polystachus leaves possesses antibacterial activities. Besides, the crude extract facilitated wound healing probably via its antibacterial activity; this justifies the traditional claimed use of the plant for treating wound and bacterial infections.*

Key words*: Acanthus polystachus, agar well diffusion, Antibacterial, excision, incision, wound healing, model*

1. Introduction

1.1. Overview of infectious diseases

Infectious diseases are among leading causes of death in the world, in the face of major medical advances. In addition, infectious diseases are the key agents for aggravating poverty in the world and causes enormous health related burden through life long disability (1). According to the WHO death projection (2013), infectious diseases will remain to be the killer diseases with a level of about 13 million human deaths annually until at least in 2030. The negative impacts of infectious diseases are highly observed in developing countries (2).

The majority of emerging infectious diseases are caused by bacteria which can be associated with evolution of drug resistant microbes. The contributing factors for emerging infectious diseases include ecological changes, human demographics behavior, microbial adaptation and change, and break down in public health measures. These changes can lead to the emergence of new diseases, and to the development of antimicrobial resistance. The new emerging infectious diseases also have a significant burden on global economies and public health(3).
Therefore, it is possible to implicate that researches focusing on the causes of infectious disease and methods of effective treatment and prevention have to be encouraged to effectively lift people out of infection and to build a better world for future generations.

1.1.1. Antimicrobial resistance

Antimicrobials are particularly valuable resources to treat and prevent infectious diseases. However, after a certain time of antibacterial usage, some bacterial pathogens became resistant to many of the first effective drugs. In addition, antibacterial agents is threatened by the dwindling supply of new antimicrobials and the global increase in antimicrobial resistance (4).
Despite the fact that the antibiotic resistance is a global threat, the burden is higher in developing countries like Ethiopia because of the high prevalence of bacterial diseases and the presence of risk factors for its emergence and spread (5). The increasing prevalence of hospital and community-acquired infections caused by multi drug- resistant (MDR) bacterial pathogens endangers for effective antibiotic therapy. Furthermore, this alarming spread of antimicrobial resistance has not been corresponding with the development of novel antimicrobials (6). Therefore, the ongoing explosion of antibiotic-resistant infections continues to be global health care problem with an equally alarming decline in the research and development of new antibiotics to deal with the threat.

The consequences of antibacterial resistance include increased morbidity, prolonged illness, a greater risk of complications and higher mortality rates. The economic burden of antibacterial resistance includes loss of productivity and increased health care cost of patients as a result of prolonged treatments, the use of expensive alternative antibiotics with serious side effects, and extended hospital stays. Generally, antibiotic resistance rates currently rising up; however, insufficient research and development activities; irrational use of antibiotics and uncoordinated overall efforts, could result in treatment of many of the infections is impossible. Thus, in order to address the impact of antimicrobial resistance on medical, social, and economical burdens, real & unreserved global coordinated efforts, paralleled with the development of new antibacterial led by the health professionals have to be taken (6, 7).

1.1.2 Causes of antimicrobial resistance

The emergence and spread of bacterial resistance have been driven by complex socioeconomic and human behavioral factors including misuse of antibiotics, the practice of practitioners and laypersons with lack of skill, and poor drug quality, particularly in developing countries. In the hospitals and community spread of resistant bacteria can be enhanced by overcrowding, lapses in hygiene or poor infection control practices. There are also various factors contributed for spread of resistant bacteria such as increasing irrational use, lack of awareness, non adherence, and use of counterfeit and substandard medications (7). Inappropriate uses of antibiotics in animals also contribute to the increase in prevalence of resistant bacteria of human significance (5, 7).

Self-medication by antimicrobials without medical guidance may result in greater probability of inappropriate, incorrect, or undue therapy which in turn can contribute to the emergence of bacterial resistance and increased morbidity. This can be hastened by the inappropriate use of antibiotics by patients and prescribers (8, 9).

1.1.3 Molecular mechanisms of antibiotic resistance

Bacteria are considered resistant to an antibiotic if the maximal level of that antibiotic that can be tolerated by the host does not halt their growth (10). The common mechanism of resistance is mainly due to the presence of outer cell membrane in gram negative bacteria and the expression of efflux pumps (10, 11), genetic changes by chromosomal mutation or by resistant gene transfer from one microorganism to other, of the same or different species (12), enzymatic degradation or modification (11, 13), permeability barriers or reduced antibiotic uptake and (14), altering the drug targets (15).

1.1.4 Strategies for combating antimicrobial resistance and bacterial infections

The antimicrobial resistance can be intervened by implementing rational use of antimicrobials and devising appropriate dosing regimens based on population-specific clinical outcomes. In addition, regulation on over-the-counter availability of antibiotics, improving hand hygiene and improving infection prevention and control are also the critical approaches for the control and prevention of emergence and spread of antimicrobial resistance (6, 16).

Cautious prescribing will reduce the emergence of bacterial resistance as it will decrease the selective pressure on bacteria. Moreover, providing local resistance data, preparing guidelines for antimicrobial prescribing, training of prescribers, intensified surveillance on antibiotic use have great roles in combating antimicrobial resistance. On top of these, a comprehensive education program to the people has to be provided to change the public ideology of antimicrobial usage; especially on the consequences of irrational uses of antibiotics (6, 10).

Generally, the rapid detection and control of spread of bacterial resistance; the development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria; development of new antibiotics and vaccines have to be implemented in order to alleviate the problems of bacterial resistance . These in turn need the collaborative efforts of all stakeholders involved in health and non-health settings including those involved in healthcare, public health, veterinary medicine, agriculture, food safety, academic, and industrial research(6, 10).

1.1.5 Treatment approaches for bacterial diseases

1.1.5.1. Conventional approaches

In order to treat the infectious diseases effectively and to reduce the emergence of bacterial resistance, the practice of antimicrobial therapy should consider the following principles of chemotherapy. These includes susceptibility of the bacteria to concentrations of the antimicrobial agent at the site of infection, the dose and route of administration of the drug, the duration of treatment and the immune status of the host. In addition, initial empirical therapy to severely diseased patients should be adequately broad spectrum and adequately dosed by considering safety and the pharmacokinetics of the drug in individual patients. For the clinical purpose, bactericidal drugs are preferred when possible as compared to bacteriostatic agents. This is because of their rapid effect on bacteria, synergistic effect when combined with other bacteriostatic drugs against bacteria difficult to eliminate residual organisms as the final elimination of the microorganism is not dependent on the host's defense mechanism (17).

1.1.5.2. Traditional approaches of managing bacterial infections

Despite enormous advances in conventional medicines, traditional medicines have been encouraged by WHO partly because some conventional drugs have failed to prove effectiveness, have serious side effects, or cannot cure certain new illnesses. About 65% of the world's populations have incorporated medicinal plants into their primary modality of health care. In Africa, 90% of the population relies on traditional healers to meet their primary healthcare needs, which indicate the presence of many medicinal plants with a potential source of new drugs(18).

The antimicrobial value of medicinal plants relies on some chemical substances produced by these plants: these chemicals called "secondary metabolites" and include alkaloids, terpenoids, flavonoids, tannins and phenolic compounds, essential oils, lectins and polypeptides, polyacetylenes. Herbal products from medicinal plants are preferred because of higher safety, efficiency, cultural acceptability and lesser side effects. However, the use of traditional plants may be associated with the problems of scarcity of valuable medicinal plants, lack of standardization of methods of preparation, poor storage conditions and incertitude in some traditional health practitioners, which affect the efficacy and the practice of traditional medicine(19, 20). Different pharmacologically important secondary active metabolites present in the leaves and root extracts of *acanthus senni, A. ilicifolius* and *A. montanus* brings the attention to look more on the medicinal importance of the *Acanthus polystachus* Delile.

1. 2. Wound and wound healing

1. 2.1. Overview of wound

Wound defined simply as the disruption of the cellular and anatomic continuity of a tissue may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue. This insult to the tissue is the portal of entry for many micro organisms and therefore, there is a need to administer substance that would heal wound rapidly to prevent the entrance of bacterial in tissues through wound openings(21).

Wound may be closed (e.g. bruises, ruptures and sprains) or open (e.g. abrasions, lacerations, avulsions, ballistics, hernias and excised or surgical wounds). Open wounds are the most common which characterized by break in the skin. Wounds can also be broadly categorized as acute or chronic wounds. An acute wound is the one that proceeds through an orderly and timely process to establish sustained anatomic and functional integrity and comprises a series of overlapping phases(22).

They are caused by external damage to intact skin and include surgical wounds, bites, burns, minor cuts, abrasions and more severe traumatic wounds such as lacerations and those caused by crush or gunshot injuries. On the other hand, chronic wounds are wounds that have not proceeded through orderly and timely reparation to produce anatomic and functional integrity after 3 months(22, 23).

1. 2.2. Wound healing

1.2.2.1. Wound healing phases and mechanisms

Wound healing processes are well organized biochemical and cellular events leading to the growth and regeneration of wounded tissue in a special manner. Healing of wounds involves the activity of an intricate network of blood cells, cytokines, and growth factors which ultimately leads to the restoration to normal condition of the injured skin or tissue. The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration (24).

Haemostasis: Tissue injury provokes immediate activation of the extrinsic and intrinsic coagulation pathways. Within minutes of injury, platelet activation products and intense vasoconstriction lead to clot formation. Vasodilatation and increase in capillary permeability follows, possibly as a result of release of histamine from activated platelets(24, 25).

This allows serum rich in proteins such as fibronectin, fibrinogen and fibrin to leak into the interstitial space, where these combine with the clot to produce a fibrin plug that temporarily closes the wound. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing.

Inflammation: The inflammatory phase is characterized by its cardinal signs: redness, warmth, swelling, pain, and loss of function. Many substances in the injured area, such as fibrin degradation products, leukotrienes, complement products, bacterial peptides, and platelet derived growth factors (PDGF) and transforming growth factor (TGF) that have been released from platelets, act as general leukocyte attractant mediators that support the recruitment of leukocytes. The neutrophils are the first blood cell types that enter the area of injury. The neutrophils engulf debris and microorganisms, providing the first line of defense against infection. After clearing any invading bacteria, the neutrophils undergo spontaneous apoptosis or are phagocytosed by wound macrophages (26, 27).

In the absence of infection, the existing monocytes differentiate into macrophages and become the major phagocytic cell at the injury site. Macrophage tasks include phagocytosis of any remaining pathogenic organisms and other cell and matrix debris. Macrophages also have a number of functions, including mediating angiogenesis, synthesizing nitric oxide and forming fibrous tissue. Thus, they are essential for the transition from the inflammatory to the repair phase because of their essential role in wound (25).

Proliferation and repair: The proliferative phase of wound healing usually occurs on the fourth day after wounding and is characterized by the early appearance of fibroblasts in the wound bed. There are 4 major steps in this phase: (i) angiogenesis, (ii) reepithelization (the process of restoring an intact epidermis after cutaneous injury), (iii) granulation, and (iv) tissue formation and collagen deposition(25, 28). Wound (tensile) strength begins to develop during this stage. Key cells for these processes are the fibroblast and keratinocyte (25, 29). Fibroblasts migrate inwards from the wound margins stimulated by many chemical activators and messengers, mostly released by macrophages, which dominate towards the end of the inflammatory phase. Fibroblasts themselves secrete a variety of cytokines, allowing other vital cells to proliferate and aid the healing process. Such cells include endothelial cells and angiocytes. Expansion of these cell numbers contributes to a process known as angiogenesis, the generation of new blood vessels(30). Hypoxia and acidosis stimulate angiogenesis. Within the wound bed, fibroblasts produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM). Fibroblasts also participate in the process of wound contraction after differentiation into myofibroblasts. Granulation occurs as the fibrin clot scaffold is replaced with new tissue rich in hyaluronan (hyaluronic acid), fibronectin and other ECM compounds. Because granulation tissue is very active metabolically and supports the proliferation of a variety of cells and proteins, it is also highly vascular. The new granulation tissue contains type I, III and V collagen fibers. Thirty percent of the collagen is type III collagen, which does not contribute to restoring tensile strength in the wound (31).

Remodeling: The tissue remodeling phase starts as early as a few days after injury and lasts up to 2 years thereafter. After the main steps of the proliferative phase are fulfilled, the density of cells, such as macrophages, keratinocytes, fibroblasts and myofibroblasts is reduced by apoptosis. Keratinocytes are the first cells to undergo programmed cell death; myofibroblasts are the second so that the accelerated proliferation and migration normalizes.

Gradually, the provisional collagen (type III initial new collagen synthesized in the proliferative phase) is replaced by the more stable collagen type I that is produced strictly oxygen dependently by fibroblasts and is deposited in a physiological alignment. The healing wound gains increased wound tensile strength. The collagen fibers contract so that the wound tissue shrinks(32, 33).

1.2.2.2. Factors affecting wound healing

There are many factors that can affect wound healing by interfering with one or more phases in wound healing process, thus causing improper or impaired tissue repair. Some of them are infection, tissue hypoxia, necrosis, exudate and excessive levels of inflammatory cytokines, delayed collagen synthesis, impaired epithelialization, and increased apoptosis and reduced angiogenesis. Other patient related factor impair wound healing includes smoking, diabetic mellitus, nutritional deficiency of vitamin C, peripheral vascular disease, medications (steroids), alcoholism and advanced age(31).

Inflammation: Although inflammation is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, preserve and aggravate many disorders. Indeed, in experimental models of repair, inflammation has shown to delay healing and to result in increased scarring. Prolonged inflammation known as chronic inflammation, a hallmark of the non-healing wound, delays healing and predisposes tissue cancer development and can lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. But, in normal situation inflammation is normally closely regulated by the body(34, 35). Drugs with anti-inflammatory activity (like steroids) are reported to delay wound healing activities. On the other hand, there are also some herbal drugs with known anti-inflammatory activity that are reported to possess wound healing activity. This can justify that a regulated and an optimum level of inflammation is essential for wound repair, whereas an unregulated and prolonged inflammation delays the wound healing processes(31).

Infection: Tissue invasion by pathogenic microbes is known as infection. Wound infections are a serious medical problem for patients with non-healing chronic wounds. The healing of these wounds is often compromised by colonisation of many different bacteria, predisposing patients to life-threatening infections. Bacterial infections are a critical component of hard-to-heal wounds, often leading to inhibition of innate inflammatory responses and resistance to therapeutics(36).

Wound infection is one of the most common diseases in developing countries because of poor hygienic conditions.

This is because wounds happen in day-to-day activities of people in various ways and are highly susceptible for infection unless they are handled in a sterile manner. Infection may prolong the inflammatory phase of the wound and thus leads to the failure of wound healing. Recurrent inflammation occurs when there is infection in wound sites. Therefore, if infection is excluded from the wound, effective healing can be achieved unless there are underling causes to the wound. Thus, antibacterial and antifungal preparations are commonly used to promote acute wound healing(31, 37).

Lipid peroxidation: Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. These cells, through their characteristic "respiratory burst" activity, produce free radicals. Thus, the wound site is rich in both oxygen and nitrogen centered reactive species along with their derivatives. The presence of these radicals will result in oxidative stress leading to lipid peroxidation (LPO), DNA breakage, and enzyme inactivation, including free-radical scavenger enzymes. Evidence for the role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be of therapeutic use in these conditions(38) (39). LPO is an important process of several types of injuries like burn, inflicted wound and skin ulcers. A drug that inhibits LPO is believed to increase the viability of collagen fibrils, increasing the strength of collagen fibers by an increase in circulation, thereby preventing the cell damage and promoting DNA synthesis. Antioxidants such as metronidazole, vitamin C, vitamin E have been shown to promote wound contraction and epithelialization(31).

Nutrition: Nutritional deficiencies can impede wound healing, and several nutritional factors required for wound repair may improve healing time and wound outcome. Vitamin A is required for epithelial and bone formation, cellular differentiation, and immune function. Vitamin C is necessary for collagen formation, proper immune function, and as a tissue antioxidant. Vitamin E is the major lipid-soluble antioxidant in the skin; however, the effect of vitamin E on surgical wounds is inconclusive. Bromelain reduces edema, bruising, pain, and healing time following trauma and surgical procedures. Glucosamine appears to be the rate-limiting substrate for hyaluronic acid production in the wound. Adequate dietary protein is absolutely essential for proper wound healing, and tissue levels of the amino acids arginine and glutamine may influence wound repair and immune function (39).

Wound hypoxia: Oxygen plays an important role in each stage of the wound healing process. It controls the migration and proliferation of fibroblasts.

Angiogenesis and leukocytes require oxygen. Therefore, oxygen delivery is a critical element for the healing of wounds. Local tissue hypoxia is a potent stimulus for the migration of fibroblasts and endothelial cells into the wound center.

However, wound healing is impeded if hypoxia persists. In an environment of 30 to 40 mm Hg of oxygen, fibroblasts cannot replicate and collagen production is severely limited. Wound hypoxia also predisposes the wound to bacterial invasion which significantly impedes wound healing.

Wound hypoxia may be worsened by many clinical conditions, such as poor cardiac output, peripheral vascular disease, diabetes mellitus, past irradiation, tobacco consumption and chronic infection(38, 40).

Formation of free radicals: Free radicals are produced by neutrophils and non-phagocytic cells. While they fight pathogens, neutrophils release inflammatory cytokines and enzymes that damage cells. One of their important jobs is to produce reactive oxygen species (ROS) to kill bacteria, for which they use an enzyme called myeloperoxidase. The enzymes and ROS produced by neutrophils and other leukocytes damage cells and prevent cell proliferation and wound closure by damaging DNA, lipids, proteins, enzyme inactivation, including free-radical scavenger enzymes, the ECM and cytokines that speed healing. Neutrophils remain in chronic wounds for longer than they do in acute wounds. Agents that demonstrate significant antioxidant activity may, therefore, preserve viable tissue & facilitate wound healing(31, 35).

Drugs: Many medications, such as those which interfere with clot formation or platelet function, or inflammatory responses and cell proliferation have the capacity to affect wound healing. Among the most frequently encountered medications/drugs that impede wound healing are systemic steroids through reduction of neutrophils and inhibition of leukocyte and tissue macrophage function, and nonsteroidal antiinflammatory drugs (NSAIDs) by inhibition of platelet function and aggregation, and chemotherapeutic drugs by inhibition of cellular metabolism, rapid cell division and angiogenesis (31).

1.2.2.3. Models for wound healing

Wound healing study can be carried out by using models like excision, incision, dead space and burn wounds (41). Each of these models has their own advantage and disadvantage and the limitation of one of these models can be solved by either of them.

Either one or all of these models may be employed for wound healing study on commonly used experimental animals like mice, rats and rabbits. A brief review of the four models in wound healing study is given below.

I) Excision wound model As the name implies, such wounds involve the removal of a significant volume of the target tissue, and the filling of the void created allows more ample material for determining biochemical and histological parameters (41, 42).

The excision site can be harvested or biopsied to obtain cells, tissue, Ribonucleic Acid (RNA), exudates, and histological specimens that have much more ample cross-sectional area and volume. If the excision covers a large enough area, serial biopsy of the wound site is feasible. Excisional wounds can be covered with occlusive dressings, which retain exudates (wound fluids) as a means of assessing the status of various soluble factors in the wound environment, such as nutrients, proteinases, cytokines, and tissue degradation products. Healing occurs from the margins and the base of the wound by the formation of a fibrin clot that is invaded by granulation tissue and by the migration of an epidermal tongue along the interface between granulation tissue and clot (eschar). Also healing rates are often monitored on the basis of total excisional volume (or cross-sectional area) filled with granulation tissue (neodermis), extent of re-epithelialization, histological organization of connective tissue, angiogenesis, and biochemical content of collagen or proteoglycans(43).

Various devices are used to generate this type of lesion in a standardized fashion, including biopsy punch, scalpel, and dermatome (set to cut very deeply or making several successive passes). The laser, if controlled precisely, can produce a type of excision with little bleeding, and the wound margins will still contain a zone of coagulation necrosis. The actual wound depth, as in partial-thickness wounds, is very dependent on species; the mouse has the thinnest skin, while the pig and other large domestic animals have a dermis that is as thick as or thicker than man (41, 44).

II) Incision wound model This wound is created by cutting of the skin horizontally or vertically with a sharp blade or scalpel. It results in rapid disruption of tissue integrity with minimal collateral damage when compared to excision wound. There is rapid extravasations of plasma and blood cells into the new tissue space and, depending on the extent and rapidity of hemostasis, the formation of a fibrin clot that bridges the injury margins. The amount of gap in the incision will depend on a number of factors, including the amount of subcutaneous fat, the tensional forces on the wound site (i.e., the orientation of the incision), and the species(45).

The skin of loose-skinned animals, such as rodents, lagomorphs, and dogs, can slide and retract over subcutaneous fascia to produce initially a large gap, while the skin of the pig, like man, is firmly attached to underlying structures and gaps little unless those fascial planes or subcutaneous structures are themselves incised(46).

Incision wounds closed by mechanical means heal rapidly with minimal scar tissue formation. But incisions that are either deliberately or accidentally left open are said to heal by secondary intention.

In this process, a much more extensive fibrin clot in the wound void eventually gives way to ample granulation tissue and a gap that is eventually bridged by epithelialization. In loose-skinned animals, wound closure is facilitated by the contraction of the margins to approximate the edges. Nevertheless, these wounds begin with several millimeters of gap and usually resolve with several tens of microns of scar tissue(47).

In this model whether the wound is bandaged, sutured, stapled, or clipped, the principle is always to reduce the tissue gap to a minimum to allow rapid and efficient bridging of the wounded edges by granulation tissue and new epithelium. As a consequence, this type of wound is excellent for biomechanical analysis of wound strength. It is less adequate for histological assessment of healing because of the limited volume/area of wound healing activity; for the same reasons, it is poor for evaluation of tissue biochemistry or epithelialization(41).

III) Infected wound model: Most *S. aureus* cutaneous wound infections models today focus on developing infection in excisional wounds topically; however, original models involved inducing the skin infection in mice by injecting a suspension of 10^7–10^9 CFU *S. aureus* subcutaneously. Within 24 hours, bacteria elicit an inflammatory response and cause localised swelling

This model of cutaneous skin infection is often modified to examine the contribution of specific skin cells or tissue structures and immune cells. This model has also evolved to include inducing damage to the skin, that is, removal of superficial keratinocytes, creation of a full-thickness excisional wound and creation of a full-thickness incisional wound or burn injury using heat. These wounds are then inoculated with 107–109 CFU of bacteria to induce infection(36).

Excision wounds involve the removal of a significant volume of the target tissue and are frequently complicated by infection and prolonged healing. A mouse model of excision wound infections was developed to establish a proof-of principle that antimicrobial could destroy infection in vivo. The model was also used to investigate antimicrobial and wound healing effects of chemicals on infected wounds & bacterial colonization(48).

Excisional wound infection models have been widely studied in models of *S. aureus* cutaneous infection.

Since, *S. aureus* is a commensal bacterium of the human skin and gastrointestinal tract; however, it is also a leading cause of cutaneous wound infections, bacteraemia, sepsis, pneumonia and endocarditis. The pathological hallmark of *S. aureus* infection is the formation of an abscess or lesion. The lack of adequate *in vivo* models of wound infection has made it difficult to investigate bacterial wound infections.

As human studies are logistically and ethically prohibitive, the use of animal models for preclinical testing of new localized or systemic therapies is the best approach to treat the problem of chronic wound infection. Also therapeutic options for both cutaneous and invasive *S. aureus* infections are becoming limited due to rising antimicrobial resistance(24, 36). Hence testing of new antimicrobial agents using relevant animal models is particularly important.

IV) Dead-space wound model: These models work by employing porous, subcutaneous implants. Connective tissue (collagen) formation serves to evaluate the wound healing pattern. All such models function by creating an artificial tissue space, though they differ in design, into which plasma infuses. This leads to development of a fibrin clot and subsequent formation of granulation tissue. Depending on the implant material, further maturation into scar may occur, and a connective tissue capsule comprised of several collagenous fascias usually surrounds the implant(49). These models are ideal for biochemical assessment because of the well-defined volume enclosed, and many of the implant materials are soft enough to be suitable for embedding in paraffin and sectioning. These implants generally have a symmetrical organization, with the least mature portion at the core, and tissue organization may be assessed simply by the histological progression of granulation tissue into the center of the implant(36).

V) Burn wound model: Burn wound is an injury, especially to the skin by fire, heat, radiation, electricity or caustic agents. Burn wound causes disruption of the skin's mechanical integrity and allows environmental microbes to cause infection and delay wound healing. Systemic infection resulting from burn wound infection is the main cause of death among patients who are hospitalized for burns. Burn wound is inflicted on appropriate animal model and wound contraction, and epithelialization periods and scar formation are used to evaluate the healing of this wound. A practicable, reliable and reproducible model for infliction of partial skin thickness burn lesions in rabbits is a possible example of burn wound model. The model is dedicated to experimental studies investigating the influence of drugs on burn wounds(41, 50).

Burn wound can be created in different ways. Round aluminum templates heated to 75ºC and applied for 5 sec to the moistened, clipped and depilated dorsal skin produced the desired depth of injury. This procedure produced the desired partial skin thickness burn injury. Also a molten wax at 80º C can be used to create a partial thickness wound. The wax is poured on the shaven back of the animal through a cylinder with circular opening. The wax is allowed to remain on the skin till it gets solidified(41).

1.2.2.4. Evaluation parameters of wound healing

Specific parameters are necessary to evaluate the progress in wound healing as well as to conclude whether the wound is healed sufficiently or not. Based on the common parameters experimental wounds are inflicted on selected animals that are classified possibly into control (negative control), standard and experimental groups and the healing process is followed up. Thus, this enables to make unbiased evaluation of the test substance for its activity on different possible groups in the experiment(51).

As far as wound experiment is concerned, there are a number of evaluation parameters for wound healing. To list some of these; rate of wound contraction, epithelialization period of the wound, the wound breaking strength (tensile strength of the wound), the collagen content of the healing wound, hydroxyproline content, histological analysis of the wound tissue in the different phases of wound healing. Which parameter is used for a single evaluation can be determined based on its convenience. For each of the wound study models discussed earlier one or more of the above parameters can be convenient for the evaluation of a wound healing process. For example, excisional wound is evaluated commonly by the rate of wound contraction and epithelialization period. Also the tissue from excisional wound scab can be extracted for further histological analysis(51, 52).

Wound breaking strength is a common evaluation parameter for incision wounds. However based on the implanted material it can also serve to evaluate the dead space wound healing. In this case the breaking strength of the granulation tissue formed on the implanted material is measured. Biochemical evaluations of wound healing like the wet and dry weights of collagen and the hydroxyproline content are suitable parameters for the dead space wound healing evaluation. Also histological analysis of the wound healing is possible in dead space wound by taking tissues formed in the implanted material(41).

1.2.2.5. Wound Management

After injury, the objective of wound healing is to restore structure and function of an injured tissue in order to approximate pre-wound characteristics. The effective management of wounds will reduce the number of complications and allow rapid return to normal function. The way in which wounds are managed affect the rate of healing, the time to return to normal function, the final cosmetic appearance and hence the satisfaction of customers.

Management of wounds depends on the stage of wound healing and can include irrigation, mechanical and chemical debridement, the use of antiseptics and antimicrobial and use of adherent and non-adherent dressing. The wound should be handled with an aseptic technique, thoroughly irrigated under adequate pressure and carefully debrided (53, 54).

1.2.2.5.1. Topical antimicrobials in wound management

The use of topical antimicrobials in wound management is controversial. They are proposed to promote normal healing by protecting the wound from superficial infection.

Potential disadvantages include expense, reduced antimicrobial spectrum, potential for bacterial resistance, creation of super-infections. Some of common antimicrobial in use and their bacterial spectrum are Cephazolin(active against Gram-positive and some Gram-negative beacteria), Bacitracin-polymxin B- neomycin (active against Gram-positive and Gram-negative bacteria, not Pseudomona spp), Silver sulphadiazinine (active against Gram-negative and some Gram-positive bacteria and fungi), Gentamicin(active for Gram-negative bacteria, and Nitrofurazone (Gram-positive and Gram –negative bacteria, not Pseudomonas spp) (53, 54).

1.2.2.5.2. Traditional medicines in wound healing

Traditional medicines include herbal medicines composed of herbs, herbal materials, herbal preparations, and finished herbal products, that contain active ingredients, parts of plants, or other plant materials, or combinations. Traditional medicines may also use animal parts and/or minerals. Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns. About 70% to 90% of populations in some industrialized nations and between 70% and 95% of citizens in the majority of developing countries use traditional medicine as primary health care to address their health-care needs and concerns. Among users of herbal remedies, more than 80% uses herbal remedies for their ailments, especially for wound management (55).

Herbal medicines are an important part of the culture and traditions of African people. Populations using traditional medicine for primary care in African countries accounts for 75% in Mali , 70% in Rwanda, 60% in Tanzania, 60% in Uganda and 90% in Ethiopia, (55). Especially traditional medicine has been practiced in Ethiopia since long time ago.

The country has about 800 species of plants that are used in the traditional health care system to treat nearly 300 mental and physical disorders (56). Several reports indicate that skin disorders are very common in Ethiopia. Thus, traditional medicine still remains the main resource for a large majority of the people in Ethiopia for treating health problems.

In general, traditional medical consultancy has a much lower cost, including the consumption of the medicinal plants required than modern medical attention(56).

Some of herbs and medicinal plants proved to be scientifically used for the treatment of cuts and wounds as a wound healer are *Gingko biloba, Centella asiatica, Nelumba nucifera, Ocimum sanctum, Eucalyptus globules(57).* Several medicinal plants are used in the Ethiopian folk medicine for wound management. One such plant is *A. polystachus* Delile

1.3. Statement of the problem

The enormous social and economic impact of wounds worldwide is a consequence of high rate of occurrence in general and their increasing frequency in the ageing population in particular. In addition to a high number of acute wounds, there are also a large number of chronic, hard-to-heal wounds associated with diseases and abnormalities that directly or indirectly culminate in damage of the coetaneous coverage, including arterial, venous, diabetic and pressure ulcers. The prevalence of chronic wounds increases with age (22).
Wounds cause pain, suffering, sepsis, infection, nausea, fatigue, depression, psychological disturbances, loss of function, loss of mobility, and personal financial cost. In many cases, wounds lead to amputation and even death(58).

Economic and social impact of wound is high and the cost of wound care is significant. The most important components are the costs of wound-related hospitalization and nursing time. In addition to the preventable human suffering and disabilities, this burden encompasses the cost of caring for disabled men, women and children; lost earnings by the patients and sometimes family caregivers; and an ongoing cycle of poverty and deprivation for poor families and societies. Social interaction may be impeded due to odour and drainage seen in some wounds. The impact of loss of self-esteem, continued pain, and possible depression is difficult to quantify, but is certainly real (59).

Numerous topical antimicrobials for wound care are available in different dosage form. Despite drugs are available, many wounds still fail to heal and remain a significant burden to patients and caregivers alike. The primary reason is antimicrobials resistance (AMR) to existing drugs with different levels of resistance. Drug resistance is a global problem affecting both developing and developed countries(60).

The resistance pattern of common wound infecting pathogen such as *S. aureas* shows increments in Ethiopia. Resistance to methicillin increased from 87.5% in 2004 to 100% in 2008 and *E. coli* resistance to beta-lactam penicillin and tetracycline increased from 60% to 77%. Antibacterial drug resistance of *E. coli* for 3rd generation cephalosporin & fluoroquinolones, and methicillin-resistant *S. aureus* (MRSA) is 85%, 90% and 86% respectively.

High rates of MRSA imply that treatment for suspected or verified severe *S. aureus* infections, such as common skin and wound infections must rely on second line drugs in many countries, and that standard prophylaxis with first-line drugs for orthopaedic and other surgical procedures will have limited effect in many settings. Second-line drugs for *S. aureus* are more expensive; also, they have severe side effects for which monitoring during treatment is advisable, increasing costs even further. High proportions of resistance to 3rd generation cephalosporins reported for *E. coli;* means that treatment of severe infections likely to be caused by these bacteria in many settings must rely on carbapenems, the last resort to treat severe community and hospital acquired infections. These antibacterials are more expensive, may not be available in resource-constrained settings, and are also likely to further accelerate development of resistance(60). The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents (61).

1.4. Literature review

The crude extracts of different medicinal plants such as *Rhamnus prinoides* leaf extract(62, 63), *Calpurnea aurea* leaf extract (64), *Peterollobium stellatum* root extract (65), *Datura stramonium, Croton macrostachyus,* and *Acokanthera schimperi* extracts(66) have been scientifically evaluated and approved for their antimicrobial activity.

The crude extracts of different species of acanthaceae family revealed antimicrobial activities such as *Acanthus ilicifolius* leaf and root extract exhibited strong inhibitory action against bacteria and fungus; the root and leaf extract of *Acanthus sennii* has antifungal, cytotoxic, anti-inflammatory, antipyretic, antioxidant, insecticidal, hepatoprotective and anti-viral effects (67); Extract from Leaves *Acanthus montanus* showed antibacterial & anti fungal activity (68, 69); Crude root extracts of *Acanthus pubescens* had antibacterial and antifungal effects (70) have been scientifically evaluated and approved for their antimicrobial activity against the growth of different pathogenic bacteria.

Recent studies revealed that *Alternanthera sessilis, Carica papaya, Catharanthus roseus, Cecropia peltata, Clerodendrum serratum Euphorbia hirta, Euphorbia nerrifolia, Ginkgo biloba, Lycopodium serratum, Morinda citrifolia, Ocimum sanctum, Pterocarpt santalinus, Sesame indicum, Rumex abyssinicus* Jacq and *Trigonella foenum-graecum* have wound healing activities (23, 71).

Moreover, studies revealed that medicinal plants such as *Dissotis theifolia* (24) & *Piper hayneanum* (48) which have antibacterial & antifungal activities also possess wound healing effects. The presence of different pharmacologically important secondary active metabolites from leave and root extracts of *A. senni, A. ilicifolius and A. montanus* brings the attention to look more on the medicinal importance of the *A. polystachus* Delile.

1.5. The Experimental plant

Acanthus polystachus Delile

Acanthus is a genus of flowering plants belongs to the family *Acanthaceae.* The *Acanthus* family is fairly large with some 2500-3000 species in about 250 genera. The family is distributed in tropical and subtropical habitats mainly around Indo-Malaysia, Africa, Brazil and Central America. The *Acanthaceae* family possesses antifungal, cytotoxic, anti inflammatory, antipyretic, antioxidant, antiviral, insecticidal, hepatoprotective, immunomodulatory, and anti platelet aggregation activities(67).

A. polystachus is shrub or small tree to 7 meter which is ecologically widespread and locally cultivated from medium to high altitudes (1000-3200m) in Moist and Wet Kolla, Weyna Dega and Moist Dega agroclimatic zones of Ethiopia (72). This species has pink flowers, soft, hairy leaves and grows slightly larger .A. polystachus Delile is native to Burundi, Rwanda, Uganda, Sudan, Ethiopia, Kenya and Tanzania. *A. puberscens* (Oliv.) Engl is used in traditional medicine for treatment of syphilis and gonnorhea in Tanzania(70).

The root extracts of *A. puberscens* (Oliv.) Engl exhibited weak antibacterial and antifungal activity. Using the brine shrimps lethality test ethanol and aqueous extracts were virtually non-toxic to brine shrimp larvae, but the dichloromethane extract exhibited mildly toxic effects.

The brine shrimp lethality test (BST) is used to predict the presence of cytotoxic activity in the extracts. An 80% ethanol extract of the leaves exhibited antifungal activity and a weak antibacterial activity and also antiviral activity against the poliovirus and measles viruses (73). The root extracts of *A.pubescens* exhibited weak antibacterial activity against Gram positive *(S. aureus, S. faecalis, S. agalactiae)* and Gram negative bacteria *(S. typhi* & *P. aeruginosa)* and antifungal activity against *Candida albicans* (70).

The roots are used for the treatment of gonorrhea and syphilis. The decoction of the leaves is used for the treatment of gastroenteritis, pneumonia and anthrax. It is also reported that a preparation of the dried leaves is used externally as a remedy for scabies in Rwanda(70).

In Ethiopia, *A. polystachus* is traditionally used for treating scorpion sting with root decoction given orally and to treat bleeding and stabbing pain leaf paste is applied. The leaves are used as a medicine with butter and applied to wounds (74).

The leaves are powdered mixed with butter pasted on the wound and exposed to sunlight for few minutes to treat wounds.

The root is crushed, squeezed with water & taken orally at night to treat trachoma (72, 74, 75). The root also used for treating malaria and intestinal worms. The roots are powdered and mixed with cold water is given orally to Dog as vaccine(74, 76).

Figure 1: Photograph of *Acanthus polystachus* Delile

1.6. Rationale for the study

Bacterial diseases are the major cause of morbidity and mortality in the world, particularly in developing countries. The current levels of irrational antibiotic consumption have led to a steady increase in antibiotics resistance. Moreover, the empirical use of antibiotics is associated with the risk of adverse reactions and the increase of antimicrobial resistance. On top of these, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and reemerging infectious diseases (77).

Efforts are being made all over the world to discover agents that can promote healing and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications. Many of the synthetic drugs currently used for the treatment of wounds are not only expensive but also pose problems such as allergy, drug resistance and this situation have forced the scientists to seek alternative drugs (47). Considering the principal drawbacks of conventional medicine, plants which are the gift from nature, provides excellent raw material for the treatment of various diseases and disorders. Hence, herbal products are often promoted to the public as being “natural” and safe alternatives to treat various diseases. Several herbs and medicinal plants proved to be a wound healers were identified and formulated for treatment and management of wounds. Various herbal products have been used in management and treatment of wounds over the years(57).

The treatment of wound and bacterial infections with traditional medicinal plants is common in the world. However, in most of the cases, these practices are handed down from generation to generation empirically without knowing the plausible mechanisms, safety, and efficacy of herbal treatments. In view of this, scientific studies have to be conducted on the traditional medicinal plants to overcome the global problem of antimicrobial resistance and for the purpose of developing a new, effective and safe antimicrobial drug and drugs that promote wound healing. The antibacterial and wound healing activity of the leaves of *A. polystachus* has not been conducted previously. Furthermore, different pharmacologically important secondary active metabolites isolated from the same family (acantheceae) such as leaves and root extracts of *acanthus senni*, *A. ilicifolius* and *A. montanus* brings the attention to look more on the medicinal importance of the *A. polystachus* Delile.

Therefore, it is necessary to study its antibacterial and wound healing activity of the plant in order to justify the traditional claimed uses for treating wound and various bacterial infections.

2. Objectives

2.1. General objective

- To evaluate the antibacterial and wound healing activities of crude and solvent fractions of the leaves of *Acanthus polystachus* Delile.

2.2. Specific objectives

- ✓ To determine the antibacterial activity of the leaf crude extracts of *A. polystachus* on selected bacteria
- ✓ To evaluate the wound healing activities of the leaf crude extract of *A. polystachus* using excision and incision model
- ✓ To evaluate the wound healing activities of the leaf crude extract of *A. polystachus* using infected wound model
- ✓ To determine the antibacterial activities of solvent fractions of *A. polystachus* on selected bacteria
- ✓ To perform prelimenary phytochemical screening test of the crude extract and solvent fractions of the leaves of *A. polystachus*
- ✓ To evaluate the acute oral toxicity of the leaf crude extracts of *A. polystachus*
- ✓ To evaluate the acute dermal toxicity of the leaf crude extracts of *A. polystachus*

3. Materials and methods

3.1. Preparation of plant material

Fresh leaves of *A.Polystachus* Delile were collected from the border of Blue Nile River (around Bahir Dar City, Amhara Regional State) and the leaves was washed and allowed to dry under the shade. The dry leaves were ground to coarse powder using mortar and pestle. The specimen was authenticated by a botanist (Mr. Abiyu Enyew), Department of Biology, College of Natural and Computational Sciences, University of Gondar, and the specimen was deposited for future reference with a voucher specimen number of WD 001.

3.2. Chemicals and reagents

The reagents and chemicals used for the study were absolute methanol (Carlo Erba Reagents, Italy), chloroform (A.R, Nice chemicals Pvt.Ltd, India), ethyl acetate (A.R, Nice chemicals Pvt.Ltd, India), distilled water; standard antibiotic discs of cefoxitin 30µg/disc (Himedia Laboratories Pvt. Ltd, India), ceftazidime 30µg/disc (Himedia Laboratories Pvt. Ltd, India), ciprofloxacin 5µg/disc (Himedia Laboratories Pvt. Ltd, India) and ampicillin 10µg/disc (Himedia Laboratories Pvt. Ltd, India). The bacteriological media that were used in the study includes Mueller Hinton agar (MHA) (Himedia Laboratories Pvt. Ltd, India), Muller Hinton Broth (MHB) (Oxoid Ltd, Basingstoke, Hampshire, England) and nutrient broth (Himedia Laboratories Pvt. Ltd, India). In addition, other agents like a readymade 0.5 McFarland standard (Remel, Lenexa Kansas 66215, USA), Dimethyl Sulfoxide (DMSO) (Blulux Lboratories Pvt.Ltd, India), sterile physiological saline (Albert David Limited, India) were used during the study.

Furthermore, Diethyl ether (Blulux Lboratories Pvt. Ltd, India), Hard paraffin (Blulux Lboratories Pvt. Ltd, India), white soft paraffin (Blulux Lboratories Pvt. Ltd, India), cetostearyl alcohol (Avishkar International Pvt. Ltd,), wool fat (S.d.fine-CHEM. Ltd, Mumbai, India), denatured alcohol (70%) (Acurex chemical manufacturing P.L.C), nitrofurazone ointment USP (0.2% w/w), (Galentic Pharma.Pvt. Ltd, India), ketamine hydrochloride (NEON Laboratories Ltd. Mumbai, India), Whatman No 1 filter paper (Schleicher and Schuell Microscience Gmbh, Germany). Ointment slab, oral gavage, shaver, permanent marker, Allies forceps, scissors, cotton swab, normal saline, graph paper, silk No 00 were used for wound healing experiments. All chemicals and reagents used were of laboratory grade.

3.3. Experimental animals

Healthy, adult Swiss albino mice of either sex (25–35g, and 6–8 weeks of age) and adult female rats (150-250 g, aged 2-3 months) was obtained and maintained in the animal house facilities at department of pharmacology, college of Medicine and health sciences, University of Gondar. They were housed individually in clean cages under standard conditions (25 ± 2 °C, 55 ± 5 % relative humidity, and 12 h light and dark cycles) and provided with pellet diet and water *ad libitum*. Animal handling and care was carried out throughout the experiment following international laboratory animal use and care guidelines (78).

3.4. Methods of Extraction

The leaves of *A. polystachus* were ground into a coarse size using mortar and pestle. Then, the coarse powder was subjected to crude extraction by maceration protocol. Briefly, one hundred fifty gram of powdered material was weighed and soaked in a flask containing 500 ml of 80% methanol in water (with a total of 1500grams powder in 5 liter solvent) for a period of 3 days with occasional shaking using a shaker at room temperature. The extract was filtered using Whatman No 1 filter paper. The residue was re-macerated for the second and third times with fresh solvent, for a total of 6 days in order to obtain a better yield.

After filtration, the three extracted solutions were combined and concentrated using a hot air oven with a temperature not exceeding 40^0C to remove the solvents. Then, the concentrated filtrate was dried by using desiccators to remove its aqueous content. Finally, the dried extract was packed in a closed vessel and stored in deep freezer until required for the experiment.

Solvent-solvent fractionation was employed by using separatory funnel. Chloroform, ethyl acetate and distilled water were used as solvents. Selections of solvents were carried out based on their increasing polarity index and immiscibility with water. First, 85 grams of the crude extract was weighed and dissolved in 250 ml of distilled water. Then it was poured into a separatory funnel into which 150 ml of chloroform was added and shaken gently. Because the density of chloroform is greater than water and also immiscible, it forms two phases with the bottom chloroform phase and the top aqueous phase so that the bottom layer was filtered and collected with beaker. Similarly, chloroform was added three times and filtered to obtain the chloroform fraction. Likewise, the chloroform marc was shaken by adding 150 ml of ethyl acetate. Since, the density of ethyl acetate is less than water it forms the top phase so that the lower phase was poured in to the beaker and the upper phase (ethyl acetate layer) was transferred in to the flask.

In this way, ethyl acetate was added 3 times and filtered to obtain the ethyl acetate fraction. The final marc left after was dried & taken as aqueous fraction. Then, the chloroform fraction, ethyl acetate fraction and aqueous fraction were concentrated in hot air oven adjusted to a temperature of not exceeding 40^{o}c to remove the solvents. Then the concentrated fractions were placed in desiccators to maintain dryness and even to remove the remaining water from aqueous fractions. Finally, the dried fractions were weighed and their percentage yields were calculated.

For preparing the desired solutions of the leaves of plant extract, dried crude extract and solvent fractions was reconstituted in 20% Dimethyl sulfoxide (DMSO) (for crude extract), 70% DMSO (for the chloroform & ethyl acetate fractions) and distilled water (for aqueous fraction) at appropriate concentration to be used for the antibacterial activity test.

3.5. Ointment formulation

Nitrofurazone ointment (0.2%w/w) was used as standard drug for comparing the wound healing potential of the extract in different animal models. Simple ointment B.P. was prepared using hard paraffin, cetostearly alcohol, white soft paraffin and wool fat.

The master formula used for the preparation of ointment was taken from British Pharmacopoeia (79) in order to prepare simple ointment base for 80% methanol extract ointment formulation.

Ingredients	**M.F**	**R.F**
Wool fat……	50 g	10g
Hard paraffin……	50 g	10g
White soft paraffin……	850g	170g
Cetostearyl alcohol……	50g	10g
	1000g	200g

M.F= Master Formula, R.F= Reduced Formula; 200 g of simple ointment base was prepared by using reduced formula. First 10 g of hard paraffin was placed in a beaker and melted over water bath. It was removed from the heat after complete melting and the other ingredients such as cetostearyl alcohol (10 g), white soft paraffin (170 g) and wool fat (10 g) was added in descending order of melting point, respectively.

All the ingredients were melted over a water bath with constant stirring until they became homogeneous. The mixture was removed from the heat and stirred until cold (80).

To prepare hydroalcoholic extract ointment, first the extract was powdered in a mortar and pestle. Then 10 g and 20 g of the powdered extract were incorporated into portion of simple ointment base to prepare 5% and 10% (w/w) ointment, respectively, by levigation on the surface of the ointment slab to make ointment of uniform consistency and smooth texture. The remainder of simple ointment base was gradually added and mixed thoroughly. Finally, the extract ointment was transferred to a clean container for topical application during the experiment (80).

3.6. Phytochemical screening

Photochemical screening test was performed on the crude extract following standard procedures (81-83).

Test for terpenoids (Salkowski test)

To 0.25 g of the crude extract of *A.polystachus* leaves was taken. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for glycosides

To 2 mL of extract 2 drops of Molisch's reagent was added and shaken well. Two milliliters of con-centrated sulfuric acid was added on the sides of the test tube. A reddish violet ring appeared at the junction of two layers immediately indicated the presence of carbohydrates.

Test for saponins

To 0.25 g of the crude extract was taken, then 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicated the presence of saponins.

Test for tannins

About 0.25 g of crude extract was boiled in 10 ml of water in a test tube and then filtered. The addition of a few drops of 0.1% ferric chloride to the filtrate resulting in blue, blue-black, green or blue-green coloration or precipitation was taken as evidence for the presence of tannins.

Test for flavonoids

About 10ml of was added to 0.25 g of the crude extract and heated on a water bath for 3 min. The mixture was cooled and filtered.

Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. Then allowed to form layers to separate and the yellow color in the ammonical layer indicates the presence of flavonoids.

Test for alkaloids: About 0.25 g of the crude extract was stirred with 5 ml of 1% HCl on a steam bath. One milliliter of the filtrate was treated with a few drops of Mayer's reagent and another 1 ml was similarly treated with Dragendorff's reagent. Turbidity or precipitation with both reagents was taken as preliminary evidence for the presence of alkaloids.

Test for polyphenols (phenolic compounds): To 5 ml of the aqueous solution of the crude extract and solvent fractions, 1 ml of $FeCl_3$ (1%) and 1 ml $K_3(Fe(CN)_6)$ (1%) were added. The appearance of fresh redish blue color indicated the presence of polyphenols.

Test for anthraquinones (Borntrager's Test)

About 0.5 g of sample of each plant extract was shaken with 5 ml of chloroform and filtered. A 10% ammonium hydroxide solution (5ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonical phase was taken as an indication of the presence of anthraquinones.

3.7. Acute oral toxicity

Acute oral toxicity test for the crude extract was performed according to the Organization for Economic Cooperation and Development (OECD) (84).

Five female albino mice of 6-8 weeks were used. All mice were fasted (food but not water) for 4 h before and 2 h after the administration of the extract. First, a sighting study was performed to determine the starting dose. For this, a single female mouse was given 2000 mg/kg of the extract as a single dose by oral gavage. There was no death observed within 24 h, additional four mice were used, and administered the same dose of the extract.

The animals were housed separately in cages and observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity, food and water intake and mortality. Observations included changes in skin and fur, eyes and mucous membranes, respiratory and behavior pattern. A special attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The change in body weight, food and water intake was recorded at two days interval(84).

3.8. Acute dermal toxicity

The test was performed according to the OECD draft guideline number 434(85). For acute dermal toxicity, a total of ten female Wister rats of age between 8 and 12 weeks were used. They were divided into two groups of five animals each for a treatment and a control groups. Animals that showed normal skin texture was housed individually in a cage and acclimatized to the laboratory condition for five days prior to the test. Following acclimation, around 10 % of the body surface area fur was shaved 24 h before the study from the dorsal area of the trunk of the test animals. First, a sighting study was performed to determine the starting dose by applying 2000mg/kg of the 10% extract ointment. There was no death or skin irritation observed within 24 h, and then additional four rats from each group were used, and applied the same dose of the extract ointment. I.e. A limit test dose of 2000 mg/kg of the 10 % ointment formulation of the extract and control groups were applied uniformly over the shaved area and observed for 24 h. At the end of the exposure period, residual test substance was removed and the animals were observed for development of any adverse skin reactions daily for 14 days (106).

3.9. Antibacterial Activity Assay

3.9.1. Test microorganisms and standard antibiotics

The antibacterial activity of the crude extract and each of the solvent fractions was tested against a set of five different bacterial strains of standard sources: Two gram positive bacteria *Staphylococcus aureus* (ATCC 25923*), Streptococcus pyogen* (ATCC 19615) and three Gram negative bacteria including *Escherichia coli* (ATCC 25922), *Klepsila pneumonia* (ATCC 1705) and *Pseudomonas aeruginosa* (ATCC 27853) were used. The antibacterial drugs used as positive controls were selected based on antibacterial spectrum, susceptibility and also by considering the availability of these agents. The standard antibiotics used as positive control were ampicillin 10 µg/disc (for *S. pyogen),* cefoxitin 30 µg/disc (for *S. aureus*), cetfazidime 30 µg/disc (for *P. aeruginosa*) and ciprofloxacin 5 µg/disc (for *E.coli & K. pneumonia*).

3.9.2. Inoculum preparation and standardization

Standard bacterial strains of *Escherichia coli, Pseudomonas aeruginosa, K.pneumonia Staphylococcus aureus* and *Streptococcus pyogens* were obtained from department of microbiology, college of medicine and health sciences, University of Gondar. Then, nutrient agar and 5% blood agar (for fastidious streptococcus species that require nutrient enriched media) was prepared following the manufacturer's protocol in a prelabelled sterile petridishes aseptically and allowed time for the congealing of the agar.

Then the bacteria was inoculated and spread on the respective prepared agar using inoculating wire loop aseptically and incubated for 24 h at 37^0c for refreshment/activation. Three to five well isolated colonies of the same morphological type of each bacterium was picked up by wire loop from fresh agar plates of bacterial culture and aseptically transferred into prelabelled test tubes containing the sterile nutrient broth and incubated for about six hours. The turbidity of the inoculums tube was adjusted visually by either adding bacterial colonies/sterile normal saline solution to that of the already prepared 0.5 McFarland standard which is assumed to contain a bacterial concentration of $1x10^8$ CFU/ml. The bacterial turbidity of each of bacterium was prepared and standardized by following the guideline of Clinical and Laboratory Standard Institute (CLSI) (86, 87).

3.9.3. Agar well diffusion

The antibacterial agar well diffusion assay was conducted to assess the presence of antibacterial activity by following the methods described previously (65, 66). Bacterial broth culture was prepared to a density of 10^8 CFU/ml of 0.5 MacFarland standards. The nutrient broth was prepared according to the manufacturer's procedure and was incubated at 37^0C for 24 h for sterility testing. Then, few colonies (3-5) of similar morphology of the respective bacteria was transferred with a sterile inoculating loop from freshly prepared agar innocula to the sterile 5 ml nutrient broth aseptically and this liquid culture was then incubated until adequate growth of turbidity equivalent to McFarland 0.5 standard was obtained. Then, The aliquot of inoculum of the respective bacteria was streaked on the sterile MHA plates (prepared according to the manufacturer's guideline) or MHA with 5% blood (for streptococcus species to enrich the medium with nutrient) in 150 millimeter (mm) diameter sterile petridish using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. Then, the plated medium was allowed to dry at room temperature for 30 minutes. On each plate, five equidistant wells were made using sterilized borer with a 6 mm diameter, depth of 5mm & 2 mm from the edge of the plate. The corresponding wells were filled with 100 µl (125mg/ml, 250mg/ml & 500mg/ml) of the solutions of the crude and each solvent fraction on the three wells and also antibiotic disc as positive control and solvent vehicle as negative control were added to the remaining wells(66). These concentrations were determined based on the data obtained from the previous study (63).

In addition, the commercial antibiotic discs of Ampicillin 10µg/disc (for *S. pyogen*), cefoxitin 30µg/disc (for *S. aureus*), ceftazidime 30µg/disc (for P. *aeruginosa)* and ciprofloxacin 5µg/disc (for *K. pneumonia* & *E. coli*) were used as a positive control; whereas, the vehicle were used as negative control(86, 87). Then, the plates were left undisturbed on the bench for about 2 h at room temperature, for pre-diffusion. Finally, the plates were incubated at 37^0c for 24h. After incubation, the diameter of the inhibition zone(s) including the diameter of the well was measured using a ruler in mm and recorded. The experiment was performed in triplicate and mean zones of inhibition was calculated for each extract and the standard antibiotics.

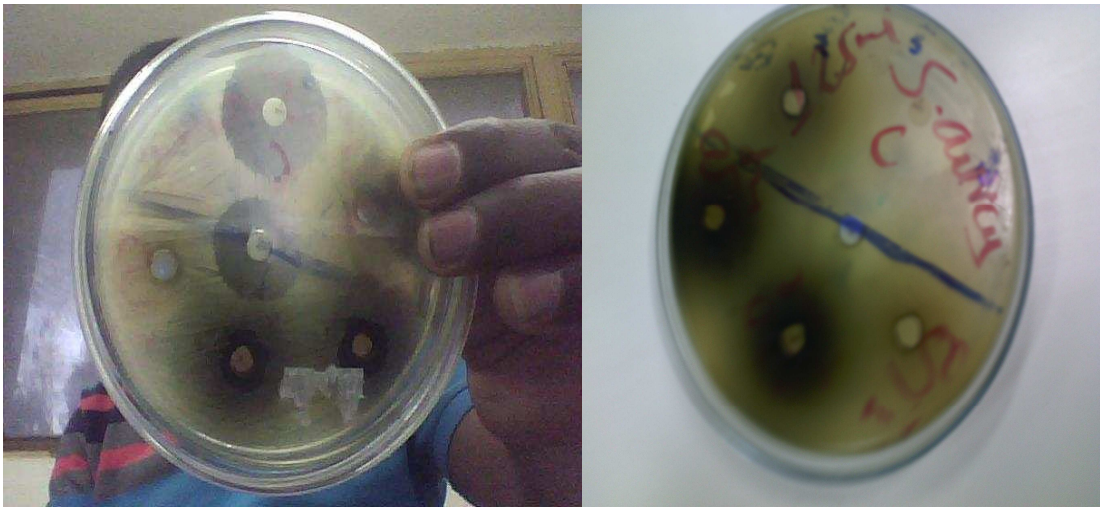

Figure 2: photograph of antibacterial susceptibility test (determination of mean zone of inhibition) against *P. aeruginosa* and *S. aureus* respectively.

3.9.4. Determination of the Minimum Inhibitory Concentration (MIC) of the Extract

The crude extract and solvent fractions that showed antibacterial activity by agar well diffusion method were subjected to serial micro broth dilution technique to determine their MIC. Under aseptic conditions, the test tubes were filled with 100 µl solution of the test materials. All the tubes were filled with 100 µl of Muller Hinton broth. Two fold serial dilution was carried out by transferring 100 µl test material from first to the subsequent test tubes in the next using micropipette so that each test tubes has 100 µl of test material in serially descending concentrations (500, 250, 125, 62.5, 31.25, 15.625). Finally, a volume of 10 µl bacterial suspension was added to each test tube to achieve a final concentration of 5×10^6 CFU/mL. To avoid the dehydration of bacterial culture, each plate was wrapped loosely with aluminum foil.

The tubes were incubated in temperature controlled incubator at 37° C for 24 h. The turbidity was then observed visually (65, 87). Any turbidity observed indicate bacterial growth and this was taken as positive. The lowest concentration of plant leaf extract at which no turbidity occurred was recorded as the MIC value. All the experiments were performed in triplicates so that the average values were taken for the MIC of test material.

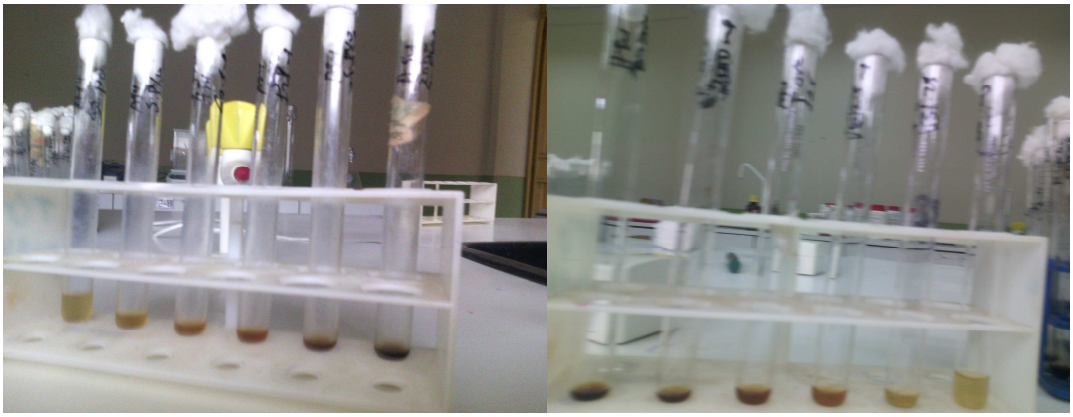

Figure 3: photograph of MIC determination of *S. aureus* and *S. pyogen* using micro-dilution

3.9.5. Determination of Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed (bactericidal concentration). This was determined from the broth dilution technique of the MIC results by sub-culturing to antimicrobial free agar. In this technique, the contents of the test tube above the MIC value was streaked using a sterile wire loop on agar plate and incubated at 37ºC for 24 h. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC(66, 88). This was performed in triplicates and the average value was taken for the MBC of test material against each bacterium.

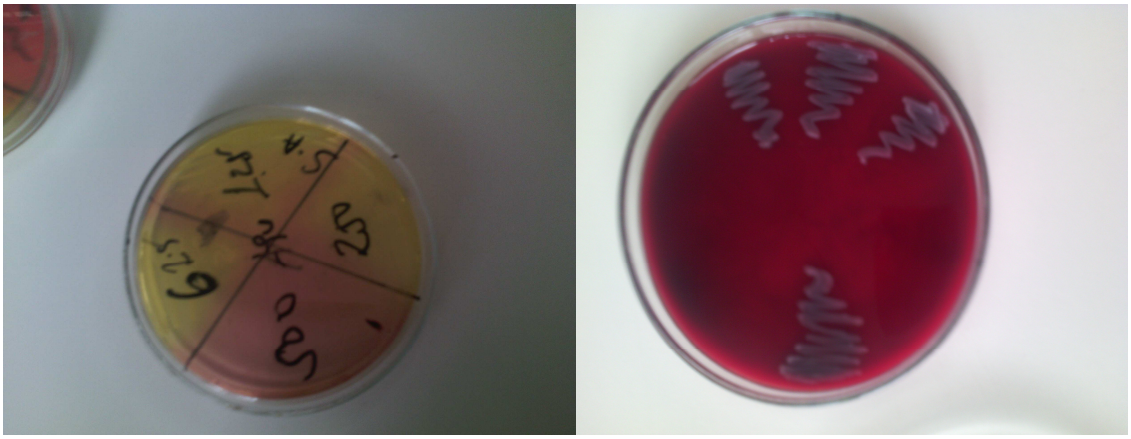

Figure 4: photograph of MBC determination against *S. aureus* and *S. pyogen* respectively

3.10. Wound healing activity testing

3.10.1. Models for wound healing activity

In-vivo excision and incision wound models were used to evaluate the wound-healing activity of leaves extract of *A.Polystachus* Delile. The healing evaluation parameters used was rate of wound closure and epitheliailization periods for excision wounds, whereas wound breaking strength for incision wounds

3.10.2. Grouping and dosing of animals

Healthy, adult white albino mice of either sex (25–35g, and 6–8 weeks of age) were used. Four groups of mice containing six in each were used for excision model. Animals in Group I was treated with simple ointment (as control) Group II was treated with nitrofurazone (0.2%) ointment (as a standard drug). Group III and group IV was treated with 5% (w/w) and 10 % (w/w) extract ointments, respectively. Four groups of mice containing six in each was used for incision wound model (24). The animals of group I-IV was treated in a similar fashion with excision wound model. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline(78)

3.10.3. Excision wound model

Excision wound was used for the study of rate of wound contraction and epithelialization period (45, 51). An impression was made on the dorsal thoracic region 1 cm away from vertebral column on the anaesthetized mouse. After wound area preparation with 70% alcohol, the dorsal fur of the animals was shaved with shaving machine and the anticipated area of the wound to be created was outlined on the back of the animals with permanent parker. A full thickness circular excision wounds sized about 300 mm^2 was created along the markings using toothed forceps, scalpel and scissors. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open.

The mice were divided into four groups (6 mice per group) randomly and each mouse was placed in a separated cage. The treatment was done once daily topically in all the cases. The wounding day was considered as day 0. The standard, extract and simple ointment were applied topically to the respective groups till the wound was completely healed (45, 51, 89).

Figure 5: photograph of excision wound on day 0, day 8 and 12 respectively

3.10.4. Infected wound model

This model was used to study the in vivo antibacterial activity and to evaluate whether infection may delay rate of wound contraction and epithelialization period (24) A full thickness circular excision wounds sized about 300 mm^2 was created along the markings using toothed forceps, scalpel and scissors using similar procedures as in the excision wound model. After achieving complete haemostasis by blotting the wound with cotton swab soaked in normal saline, the wound was inoculated with standard strain of *S. aureus* culture (24 hrs) obtained from department of microbiology, school of medicine and health sciences, University of Gondar. After 24 h of contamination with *S. aureus* suspensions (1x 10^6 CFU/mL), the experimental animals were randomized into four groups (1-4). The animals were placed singly in individual cages and treated in similar procedures followed as in the case of excision wound model. Treatments of infected wounds commenced on the 2^{nd} day to allow establishment of infection on the wound. The wound area was measured with a translucent paper and a 1 mm^2 graph sheet on day 0, 2, day 4 and thereafter every other day until completed wound closure was recorded. Wound contraction was calculated as a percentage of the original wound size (24, 48). Throughout the experiment, presence or absence of phlogistic characteristics (infiltration, edema/localized swelling, abscess or lesion and exudates) were monitored every 24 hours (48).

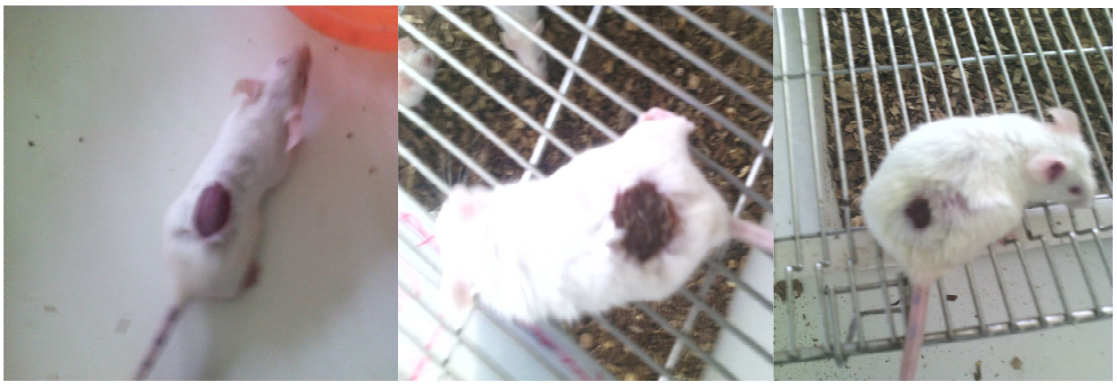

Figure 6: photograph of excision infected wound on day 0, day 2 & 10 respectively

Measurement of wound contraction

The wound closure rate was assessed by tracing the wound on days 2, 4, 6, 8, 10, 12, 14 and 16 post wounding days using transparent paper and a permanent marker. In case of infected wound model measurement of wound contraction was prolonged till 18 days. The wound areas recorded was measured using $1mm^2$ scale of graph paper. Changes in wound area were evaluated, giving an indication of the rate of wound contraction and epithelialization period. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound as 100 % (51) as shown below:

$$\textbf{\% Wound closure} = \left(\frac{\text{wound area on } 1^{st} \text{ day} - \text{Wound area on day (n)}}{\text{Wound area on } 1^{st} \text{ day}}\right) \times 100$$

Where n = number of days (2^{nd} , 4^{th} etc.)

Epithelialization period measurement

Falling of scab leaving no raw wound behind was taken as end point of complete epithelialization and the days required for this was taken as period of epithelialization(51).

3.10.5. Incision wound model

Animals were anesthetized in the same manner described for excision wound model. The dorsal fur of each mouse then shaved and a 3 cm long longitudinal paravertebral incision 1cm away from vertebral column was made through the skin and subcutaneous tissue. The parted skin was then sutured 1 cm apart using a surgical thread (silk no. 00 round) as described by Ehrlich and Hunt with slight modification(46, 49). After 24 h of wound creation (on 1st day), animals was treated as described under grouping section, with topical formulation of non-medicated simple ointment, extract and standard drug once daily for nine days. The suture was removed on day 8 post-incision and tensile strength was measured on the 10th post-wounding day using continuous water flow technique (49, 90).

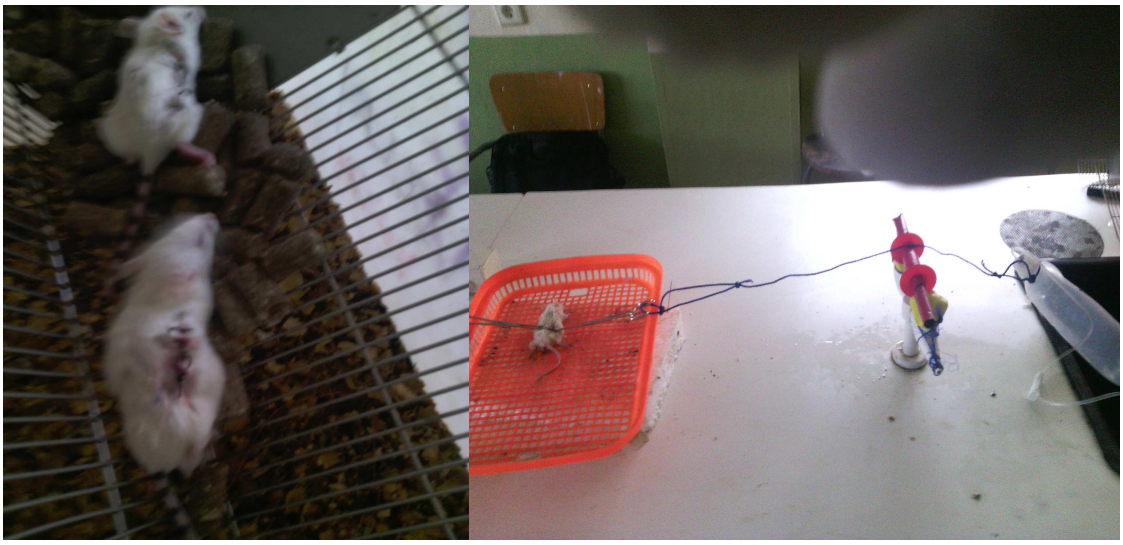

Figure 7: photograph of Incision wound day 0 and measurement of tensile strength on 10th day using water flow technique

Measurement of tensile strength

Tensile strength (the force required to open the healing skin) was used to measure the extent of wound healing. The model used for this purpose consists of fixed shelves with a table. Two Allis forceps, one is fixed to the opposite side of shelve and another is tied with and hanged with rope that was attached to the empty IV bag on which the weights are placed. On the 10th post-wounding day each mouse was anaesthetized using diethyl ether to secure animal to the table. The two forceps was firmly applied 1cm away from healed tissue on the incised part of the skin on to the line facing each other. Water is allowed to flow into bag from tap water through IV line. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. As soon as wound gaping appeared, water flow was stopped, and the volume of water collected in the container was determined and noted as an indirect measure of breaking strength in grams. Percentage of tensile strength for extract and reference drug with respect to negative control treated with simple ointment (SO) was measured using the following formula (49).

$$\text{Tensile strength (TS) of extract \%} = \frac{\text{TS extract} - \text{TS}_{\text{S.O}}}{\text{TS}_{\text{S.O}}} \times 100$$

$$\text{Tensile strength (TS) of standard \%} = \frac{\text{TS standard} - \text{TS}_{\text{S.O}}}{\text{TS}_{\text{S.O}}} \times 100$$

3.11. Ethical clearance

Ethical approval was obtained from scientific research and Ethics Committee of the college of medicine and health sciences, University of Gondar.

3.12. Data management, processing and analysis

The data was entered, coded, cleared, processed and analyzed using Statistical Package for the Social Sciences (SPSS) version 20. The experimental data was expressed as mean ± Standard Error of the Mean (SEM). Finally, one way analysis of variance (ANOVA) followed by Tukey Post Hoc Multiple Comparison test was employed and P value $P<0.05$ considered statistically significant.

4. Results

4.1. Crude extraction and fractionation

Maceration technique was used for extraction. About 1.5 grams of the leaf powder was subjected for crude extraction (80% methanol) and the yield valve was 14%. Then, 85 grams of crude extract dried powder was measured and employed for solvent-solvent fractions. The yield value for fractionation was ethyl acetate fraction 11.76% (10 gm), chloroform fraction 17.65% (15 gm) and aqueous fraction56.5% (48 gm).

4.2. Acute toxicity

4.2.1. Acute oral toxicity test

There was no mortality observed in animals through the 14-day period following single oral administration at dose level of 2000mg/kg of the crude extract of *A. polystachuis* leaves. Morphological characteristics (fur, skin, eyes, and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy, or unusual behaviors such as self-mutilation and walking backward were observed; gait and posture, reactivity to handling or sensory stimuli, and grip strength were all normal.

4.2.2. Acute dermal toxicity

Selection of topical base was important to prepare topical formulations with negligible risk of skin irritation, optimum flow, spreadability and release properties. The developed ointments were stored in tightly closed containers and evaluated for acute dermal irritation test. Maximum concentration of hydro-alcohol ointment (10% w/w) applied using a limit dose of 2000mg/kg of body weight was found to be safe. After 4h and 24 h, the application site did not show any sign of inflammation and edema. There were no signs of toxicity seen when the animals were monitored for 48 h. There was also neither mortality nor any sign of toxicity observed in rats when monitored for 14 days after topical application of the extract.

4.3. Phytochemical constituents of the crude extract and solvent fractions

According to the qualitative phytochemical screening study, the crude extract of the leaf of *A. Polystachus* Delile was found to be positive for the presence of all of the tested secondary metabolites except for, alkaloids and steroids; whereas the test of glycosides was positive only in the crude extract and chloroform fraction. However, the presence of tannins, flavonoids, saponins, polyphenols and anthraquinones was confirmed in the ethyl acetate and aqueous fractions.

Table 1: Preliminary phytochemical screening of the crude extract and the solvent fractions of leaves of *A. Polystachus* Delile using chemical test methods

Metabolites tested	Crude extract	Solvent fractions		
		Chloroform	Ethyl acetate	Aqueous
Tannins	+	_	+	+
Saponins	+	+	+	+
flavonides	+	_	+	+
alkaloides	_	_	_	_
Polyphenols	+	+	+	+
Anthraquinones	+	+	+	+
terpenoides	+	+	+	_
Glycosides	+	+	_	_
Steroids	_	_	_	_

Note: + = present, - =absent

4.4. Antibacterial activity

4.4.1. Agar well diffusion method

The results of antibacterial activity test using agar well diffusion technique revealed that the growth of all test bacterial strains (except *E .coli*) were inhibited by all the tested concentrations (125mg/ml, 250mg/ml & 500mg/ml) of the crude (80% methanol) extract of the plant in concentration dependent manner. Among the test bacteria, gram positive bacterial species (*S. aureus, S. pyogen)* and gram negative bacteria *(P. aeruginosa)* were more susceptible than that of the other gram negative bacterial species at the corresponding tested concentrations of the crude extract, especially at 500 mg/ml and 250 mg/ml. As depicted in Table 2, the most susceptible bacterium at 500 mg/ml was standard strain of *S. aureus*, standard strains of *S. pyogen* followed by *P. aeruginosa* with a mean zone of inhibition of 16.33 mm, 12.33 mm and 8.00 mm, respectively.

Moreover, the crude extract mean zone of inhibition at 125 mg/ml was statistically significant ($p<0.05$) compared to mean zone of inhibition at 250 & 500 mg/ml against the growth of *S.aureus & S.pyogen.* The crude extract also showed statistically significant antibacterial activity against standard strain of *K. pneumonia* at 250 & 500 mg/ml with the maximum mean zone of inhibition of 7.67mm &10.67 mm, respectively. However, the gram negative bacteria (*K. pneumonia* and *P. aeruginosa)* were relatively less susceptible compared to the gram positive bacterial species at comparable concentration level of the crude extract.

E. coli was not found to be susceptible against all tested concentrations of crude extract, chloroform fraction, ethyl acetate fraction and aqueous fractions (Table 2*)*.

Among the solvent fractions, the chloroform fraction was devoid of an antibacterial activity against any of the test bacterium with the exception of *K. pneumonia*. However, the ethyl acetate and the aqueous fractions were endowed with antibacterial activities. The antibacterial activity pattern of ethyl acetate fraction seemed to be in line with that of the aqueous fractions, especially in terms of its antibacterial spectrum. The most susceptible bacteria against ethyl acetate fraction was standard strains of *S. pyogen* followed by standard strains of *P. aeruginosa* with a maximum mean zone of inhibition of 14 mm and 11.67 mm, respectively, at 500 mg/ml concentration. Furthermore, the zones of inhibition of the aqueous fraction at 125 mg/ml showed statistically significant difference effects compared to 500 mg/ml concentration against each of the test bacterium ($P<0.05$) (Table 2).

Furthermore, the observed mean zone of inhibition of the crude extract, chloroform, ethyl acetate and aqueous fractions at the tested concentrations were showed statistically significant difference compared to that of their respective positive control ($p<0.05$) against all test (susceptible) bacteria. The zone of inhibition of the crude extract was greater than that of the ethyl acetate fraction at equal concentrations against the growth of each test bacterium, with a significant difference ($p<0.05$) against *S. aureus* (at 250mg/ml &500mg/ml concentrations), *S. pyogen* strains (at 500 mg/ml) and *P. aeruginosa* (at 125 mg/ml & 500mg/ml).

The zones of inhibition of aqueous fraction at 250 mg/ml and 500mg/ml concentrations were also significantly significant ($P<0.05$) compared to that of the crude extract at respective concentrations against each of the test bacterium for which it was able to inhibit their growth. Similarly, the zone of inhibition of the chloroform fraction was comparable to the crude extract at their respective concentrations' against the growth of *K. pneumonia* (Table 2). Generally, the antibacterial activities of the chloroform fraction were lower than that of the crude extract, ethyl acetate and aqueous fractions.

Table 2: Zone of inhibition (in mm) of the different concentrations of crude extract and solvent fractions of the leaves of *A. polystachus Delile* against gram positive and negative bacteria

Test category	concentrations	Bacteria species				
		S. aures	*S. pyogen*	*P. aeruginosa*	*K. pneumonia*	*E. coli*
crude extract	125mg/ml	10.67 ±0.33 ^{a3c2d3}	8.00 ±0.00 ^{a3c2d3}	--	---	---
	250mg/ml	15.00 ±0.00 ^{a3d3}	10.33 ±0.33 ^{a3d2}	7.67 ±0.33 ^{a3}	7.67 ^{a3d3}	---
	500mg/ml	16.33 ±0.33 ^{a3}	12.33 ±0.33 ^{a3}	8.00 ±0.00 ^{a3}	10.67 ^{a3}	---
	Cef.30µg/disc	22.00 ±0.00	NT	NT	NT	NT
	Amp.10µg/disc	NT	30.33 ±0.33	NT	NT	NT
	Ceft. 30µg/disc	NT	NT	15.33 0.33	NT	NT
	Cip. 5µg/disc	NT	NT	NT	20.33	20.00 ±0.00
chloroform fraction	125mg/ml	---	---	---	8.33 ±0.33 ^{a3c2d3}	---
	250mg/ml	---	---	---	10.00 ±0.00 ^{a3d3}	---
	500mg/ml	---	---	---	13.33 ±0.33 ^{a3}	---
	Cef.30µg/disc	16 ±0.00	NT	NT	NT	NT
	Amp.10µg/disc	NT	30.33 ±0.33	NT	NT	NT
	Ceft.30µg/disc	NT	NT	15.67 ±0.67	NT	NT
	Cip. 5µg/disc	NT	NT	NT	20.33 ±0.33	20.33 ±0.33
ethyl acetate fraction	125mg/ml	7.33 ± 0.33 ^{a3c1d3}	8.00 ^{a3c3d3}	7.33 ± 0.33 ^{a3c1d2e3}	---	---
	250mg/ml	9.00 ±0.00 ^{a3d1f3}	10.67 ^{a3d3}	10.00 ±0.57 ^{a3}	---	---
	500mg/ml	11.00 ±0.00 ^{a3g3}	14.00 ±0.00 ^{a3g2}	11.67 ±0.88 ^{a2g1}	---	---
	Cef.30µg/disc	20.67 ±0.33	---	---	NT	NT
	Amp.10µg/disc	NT	30.67 ±0.67	NT	NT	NT
	Ceft. 30µg/disc	NT	NT	15.00 ±0.00	NT	NT
	Cip. 5µg/disc	NT	NT	NT	20.33 ±0.33	20.00 ±0.00
aqueous fraction	125mg/ml	---	9.00 ± 0.58 ^{a3c3d3}	7.33±0.33 ^{a3c1d2e3}	---	---
	250mg/ml	7.33 ±0.33 ^{a3d3f3}	12.00 ± 0.00 ^{a3d2f1}	9.67±0.67 ^{a3}	---	---
	500mg/ml	10.67 ±0.33 ^{a3g3}	14.00 ±0.00 ^{a3g2}	11.67±0.88 ^{a1g1}	---	---
	Cef.30µg/disc	22.00 ±0.00	NT	NT	NT	NT
	Amp.10µg/disc	NT	30.67 ± 0.33	NT	NT	NT
	Ceft. 30µg/disc	NT	NT	14.67±0.33	NT	NT
	Cip. 5µg/disc	NT	NT	NT	20.67 ±0.33	21.67 ±0.33

Note: Values are expressed as Mean ± S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared to positive control, ^{b} to 125mg/ml, ^{c} to 250mg/ml, ^{d} to 5000mg/ml, ^{e} to crude 125mg/ml, ^{f} to crude 250mg/ml, ^{g} to crude 500mg/ml; $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$;Ceft=Ceftazidime, Cef=Cefoxitin, Amp=Ampicillin, Cip =Ciprofloxacin, --- = the extract lacks antibacterial activity, NT=not tested. The negative control has shown no antibacterial activity.

Minimum inhibitory concentration of crude extract and solvent fractions

As presented in Table 3, the MIC values of the crude extract were in agreement with its preliminary antibacterial activities i.e the more susceptible is the bacterium, the lower is the concentration of the extract required for growth inhibition in most of the test bacteria. The crude extract of the plant was more potent against gram positive bacteria than that of gram negative bacteria. The MIC for the crude extract was 26.04mg/ml & 31.25mg/ml (against *S. aureus & S. pyogen*). Whereas, the MIC value of the crude extract against both gram negative bacteria (*P. aeruginosa & K. pneumonia*) was 125mg/ml. In the case of the ethyl acetate fraction the MIC value was 31.25 mg/ml & 52.08 mg/ml against *S. aureus & S. pyogen* respectively. Similarly, the MIC value in the aqueous fractions was 62.5 mg/ml & 125mg/ml against *S. pyogen* & *S. aureus* respectively. The MIC value of the chloroform fraction against *K.pneumonia* was found to be 125 mg/ml.

Table 3: The MIC (in mg/ml) of the crude extract and the solvent fractions of the leaves of *A. polystachus* Delile against gram positive and gram negative bacteria

Bacteria	Extract category			
	Crude	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
	MIC	MIC	MIC	MIC
S. aureus	26.04 ±5.21	__	31.25 ±0.00	125 ±0.00
S. pyogen	31.25 ±0.00	__	52.08 ±10.42	62.5 ±0.00
P. aeruginosa	125.00 ±0.00	__	125.00 ±0.00	250 ±0.00
K .pneumonia	125.00 ±0.00	125.00 ±0.00	__	__

Note: MIC= Minimum Inhibitory Concentration, --- = does not have activity, the values are the average of triplicate tests.

Minimum bactericidal concentration of crude extract and solvent fractions

Based on the MBC determination method, the crude extract and active solvent fractions of the *A. polystachus* leaves were found to be bactericidal. However, the crude extract showed bactericidal activity at lower concentrations compared to each of the solvent fractions.

The maximum MBC for the crude extract was 250 mg/ml (against *P. aeruginosa*) and the minimum value was 125 mg/ml (against *S.aures*). The corresponding values of ethyl acetate fraction were 500 mg/ml (against *S. aureus* & *S. pyogen*) and 250mg/ml (against *P. aeruginosa, S. pyogen & K. pneumonia*). Similarly, the mean MBC values of the aqueous fraction were range from 500 mg/ml (against *S. aureus* & *P. aeruginosa*) to 250 mg/ml (against *S. pyogen*). In general, the crude extract was more potent and killed the bacteria at lower concentration compared to that of the ethyl acetate and aqueous fractions (Table 4).

Table 4: The MBC (in mg/ml) of the crude extract and the solvent fractions of *A. polystachus* Delile against gram positive and gram negative bacteria

Bacteria	Extract category			
	Crude	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
	MBC	MBC	MBC	MBC
S. aureus	125 ±0.00	__	500±0.00	500±0.00
S.pyogens	250 ±0.00	__	500±0.00	250 ±0.00
P. aeruginosa	250±0.00	__	250±0.00	500±0.00
K.pneumonia	250 ±0.00	250 ±0.00	__	__

Note: MBC=Minimum Bactericidal Concentration, --- = does not have activity, the values are the average of triplicate tests

4.5. Wound healing activity

4.5.1. **Excision model**

Wound contraction

Topical applications of ointments of the 80% methanolic extracts of *A. polystachus* leaves showed significant effect on wound healing process in mice. The progress of wound contraction induced by treatment of 5% (w/w) and 10% (w/w) ointment of 80% methanolic extract, simple ointment base and nitrofurazone 0.2 %(w/w) ointment is shown in Table 5. The plant extracts facilitated wound contraction significantly at both dose levels from 4^{th} day to 16^{th} day as compared to negative control. The 10% (w/w) 80% methanolic extract ointment treated group showed significant ($p< 0.05$) wound contraction starting from day 4. This effect was highly significant ($p< 0.001$) from 6^{th} day onward in comparison with the control group (simple ointment). There was no significant difference in wound healing activity between the 10% (w/w) and 5% (w/w) extract, but higher rate of wound closure was observed with 10 % (w/w) ointment. The maximum percentage (rate) of wound contraction was observed in animals treated with 10% extract ointment from the 10^{th} to 14^{th} day which was 92.14 % and 100%, respectively. Whereas, similar percentage of wound contraction (92.18% & 100%) was observed in animals treated with the standard drug from the 12^{th} to 16^{th} day. Hence, the 10% extract ointment revealed better observable effect compared to the standard drug; however, failed to reach statistical significance.

The animals treated with 5% (w/w) methanolic extract ointment showed significant ($p<0.05$) wound contraction from 6^{th} day onward as compared to control group. Significant ($p < 0.01$) wound contraction was also observed for nitrofurazone 0.2 % (w/w) ointment treated group from 6^{th} day onward as compared to control group. The maximum percentage of wound contraction for nitrofurazone 0.2% (w/w) ointment was seen in the 12^{th}, 14^{th} and 16^{th} day which was 92.18, 98.39% and 100% respectively. However, there was no significant difference in wound healing activity between the 10%, 5% extracts and the standard drug. Furthermore, complete wound closure was observed in standard & 10% (w/w) extract ointment treated groups within 14 &16 days respectively (Table 5).

Table 5: Effect of topical application of the 80% methanolic extract of the leaves of *A. polystachus* on wound contraction of excision wound model in mice

Treatments		SO	NF	5 % CEO	10% CEO
Wound area (mm^2)- post wounding days	0	301.21 + 6.14	301.21+ 6.14	298.70+ 6.84	301.21+ 6.14
	2	278.81+ 9.13 (7.51%)	264.55+12.29 (12.29%)	273.71+ 6.12 (8.29%)	264.02+ 6.12 (12.23%)
	4	240.61+ 6.14 (20.07%)	210.38+12.71 (30.42%)	222.81+ 8.16 (25.28%)	209.60+ 5.46 (30.39%)
	6	214.18+ 8.62 (28.85%)	155.96+15.43a2 (48.60%)	165.51+ 7.55[a1] (44.64%)	123.63+ 9.97[a3c1] (59.12%)
	8	169.56+9.56 (43.87%)	91.32+11.85 [a3] (69.87%)	98.78+ 8.57 [a3] (67.10%)	64.89+ 8.17 [a3] (78.65%)
	10	130.71+12.31 (56.87%)	48.80+ 4.65 [a3] (83.87%)	52.99+ 5.05 [a3] (82.39%)	24.08+ 5.39 [a3c1] (92.14%)
	12	94.07+12.43 (69.01)	23.81+4.76 [a3] (92.18%)	23.68+47.09 [a3] (92.27%)	3.40+ 1.29 [a3] (98.91%)
	14	50.89+10.02 (83.14%)	4.97+1.99 [a3] (98.39%)	7.07+3.18 [a3] (97.73%)	0.00+0.00 [a3] (100%)
	16	31.01+ 5.38 (89.74%)	0.00+ 0.00 [a3] (100%)	1.70+ 1.18 [a3] (99.46%)	0.00+ 0.00 [a3] (100%)

SO, simple ointment base; CEO, Crude Extract Ointment; NF, nitrofurazone, n = 6 animals in each group; Values are expressed as mean ± S.E.M (*n* = 6); one way ANOVA. [a] against control, [c] against 5% (w/w) hydroalcoholic extract, [1]*P <0* .05, [2]*P < 0*.01,[3]p<0.001 .

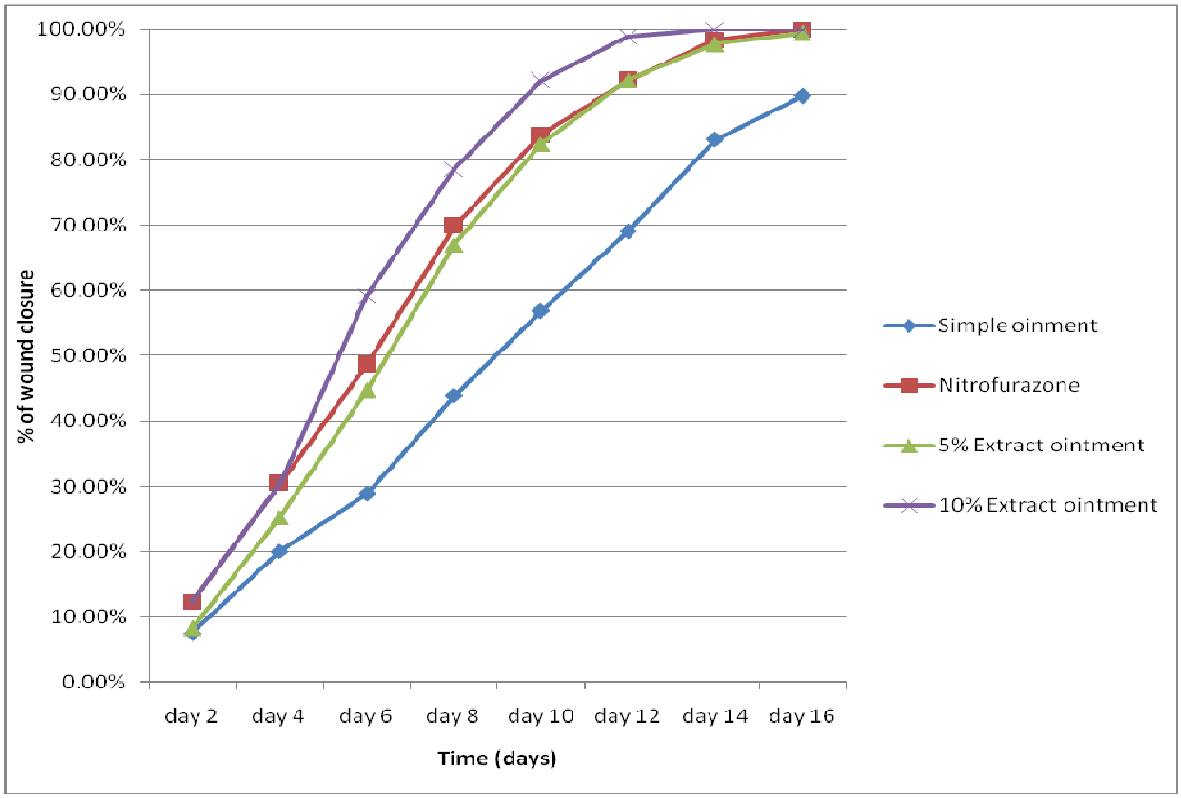

Figure 8: Effects of the 80% methanolic extract of *A.polystachus D. leaves* on the percentage wound closure of excision wound model in mice.

Epithelialization period

The time for complete epithelialization was short in extract ointment and nitrofurazone treated groups as compared to control (simple ointment treated group). On average the period of epithelialization was 20.83, 15.33, 15.83 and 13.17 for control group, standard drug, 5 % (w/w) and 10% (w/w) extract ointment respectively. The 10% extract ointment treated group showed faster rate of epithelialization ($p<0.001$) compared to control group. Similarly, 10% (w/w) extract ointment showed significant ($p<0.05$) difference of epithelialization period as compared to 5% (w/w) extract ointment treated group. Moreover, the 10% (w/w) extract showed higher percentage decrease in epithelialization periods than nitrofurazone but failed to reach statistical significance. Furthermore, comparing treatment groups by percentage decrease in epitheliliazation periods. Animals treated with 10% (w/w) extract and nitrofurazone ointments showed significant decrease (36.77% and 26.4%) in epithelialization period ($P<0.001$) respectively. Likewise, 5% (w/w) ointment treated group exhibited significant decrease (24%, $p< 0.001$) in epitheliliazation period as compared to control group. However, the difference

between epitheliliazation periods of the extract ointment treated groups with standard drug treated group was failed to reach statistical significance (Table 6).

Table 6: Effect of topical application of the 80% methanolic crude extract ointment of the leaves of *A.polystachus D.* on Period of epithelialization (no. of days)

Treatment groups	Period of epethelialization (days) Mean ± SEM	% decrease in epethelialization periods
Simple ointment base	20.83 ± 1.01	-
Nitrofurazone (0.2% w/w)	15.33 ± 0.42[a3]	26.4%
5% crude extract ointment (w/w)	15.83 ± 0.65[a3]	24%
10% crude extract ointmetnt (w/w)	13.17 ± 0.40 [a3c1]	36.77%

Note: Values are expressed as mean ± S.E.M (n = 6); one way ANOVA. [a] compared to negative control(simple ointment), [c] compared to 5% ointment; [1]$P < 0.05$, [2] $p<0.01$, [3] $P <0$ .001, when compared to control group; one way ANOVA.

4.5.2. Infected wound model

Wound contraction

In this experiment, before treatment the wounds in all animals were exhibited phlogistic characteristics (infiltration, blister formation, edema and exudates). These characteristics were vanished in the groups treated with 10% extract ointment, 5% extract ointment & nitrofurazone (0.2%) ointment within 4 to 6 days of treatment. Whereas, the group treated with simple ointment exhibited these phlogestic characteristics for one weak and above. After follow up period the negative controls were treated with nitrofurazone ointment but three of the mice were passed away in 19 & 21 days possibly due to infections.

The ointments of crude extract revealed significant effect of wound healing in mice infected with *S. aureus*. The rate of wound healing was faster for the groups treated with 10% as compared to nitrofurazone and 5% ointment. Wound area contraction was promoted and completely healed within 16 & 18 days for group treated with 10% extract followed by standard (nitrofurazone) & 5% extract ointment treated groups. The 10% (w/w) extract ointment treated group showed significant ($p< 0.05$) wound contraction starting from day 4, and statistically highly significant ($p< 0.001$) difference was observed from 6th day onward in comparison with the negative control group. The maximum percentage of wound contraction was seen from 12, 14 and 16 days which were 97.67, 99.44 and 100% respectively.

Likewise, animals groups treated with 5% (w/w) extract ointment and nitrofurazone showed significant ($p<0.05$) wound contraction from 4th day onward as compared to negative control group. The maximum rate of wound closure for nitrofurazone 0.2% (w/w) ointment was seen on the 12th, 14th, 16th and 18th days which was 93.54, 98.17%, 99.97% and 100% respectively. Even though, higher rate of wound closure was observed with 10 % (w/w) ointment, there was no statistically significant difference between the 10% &5% extracts and the standard drug.

Table 7: Effect of topical application of 80% methanolic crude extract ointment of the leaves of *A .polystachus on* wound contraction of infected wound model in mice.

Treatments		SO	NF	5 % CEO	10% CEO
Wound area (mm^2)- post wounding days	0	298.20 ± 6.84	301.21 ± 6.15	298.69 ± 6.84	301.21± 6.15
	2	278.81± 9.13 (6.67%)	259.45 ± 8.73 (13.91%)	268.87 ± 6.50 (10.00%)	259.19 ± 4.84 (13.84%)
	4	254.87± 10.32 (14.62%)	205.81 ± 10.09 [a2] (31. 88%)	213.92± 5.79 [a1] (28.42%)	201.22± 6.49 [a2] (33.06%)
	6	214.44± 11.01 (28.24%)	144.31± 11.38 [a2] (52.31%)	150.86± 8.81 [a2] (49.68%)	126.91± 9.81 [a3] (57.86%)
	8	186.57± 17.40 (37.66%)	85.04± 8.72 [a3] (71.92%)	96.03± 9.23 [a3] (67.97%)	67.38± 8.47 [a3] (77.63%)
	10	156.22± 17.46 (47.84%)	43.70± 6.69 [a3] (85.54%)	56.00± 8.15 [a3] (81.36%)	27.74± 6.98 [a3] (90.82%)
	12	120.24± 19.51 (59.96%)	19.63± 5.73 [a3] (93.54%)	27.09± 8.02 [a3] (91.06%)	7.07± 3.18 [a3] (97.67%)
	14	107.02± 18.87 (64.22%)	5.63± 3.14 [a3] (98.17%)	11.12± 5.52 [a3] (96.40%)	1.70± 1.19 [a3] (99.44%)
	16	85.31± 18.59 (71.42%)	0.66± 0.51 [a3] (99.79%)	2.49± 3.56 [a3] (99.20%)	0.00± 0.00 [a3] (100%)
	18	68.04± 16.99 (77.13%)	0.00± 0.00 [a3] (100%)	0.00± 0.00 [a3] (100%)	0.00± 0.00 [a3] (100%)

SO, simple ointment base; CEO, Crude Extract Ointment; NF, nitrofurazone, n = 6 animals in each group; Values are expressed as mean ± S.E.M (n = 6); one way ANOVA. [a] against negative control, [1]$P < 0.05$, [2]$P < 0.01$,[3]$p<0.001$.

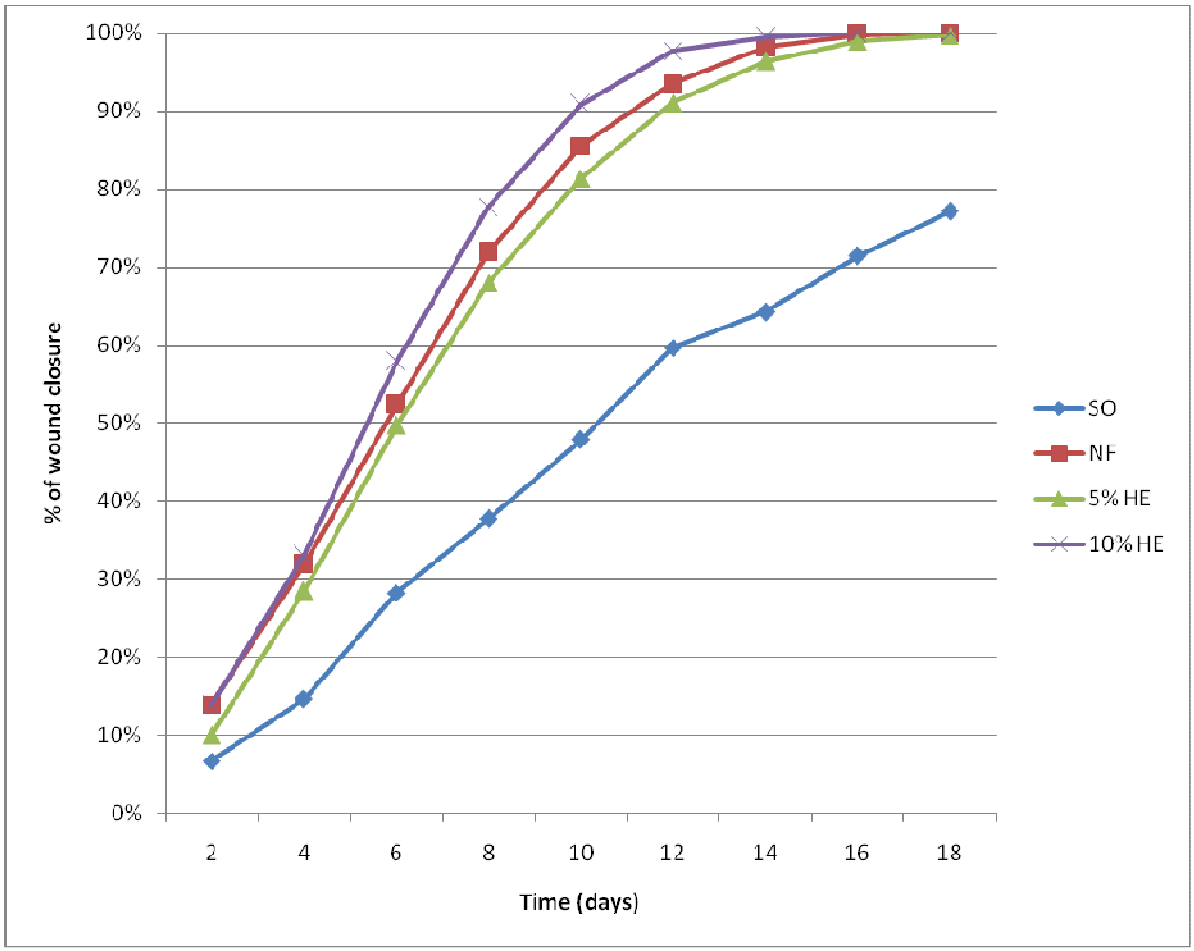

Figure 9: Effects of 80% methanolic crude extract ointment of *A .polystachus* leaves on percentage wound closure of infected wound model in mice.

Epithelialization period

In the case of infected wound model, the period of epithelialization was short in extract ointment and nitrofurazone treated groups as compared to negative control. The epithelialization period was reduced in a dose-related manner from 17.00 ± 0.86 for the 5% ointment to 14.17 ± 1.17 for the 10% ointment treated groups. On average, the period of epithelialization was 16.17, 17.00 & 14.17 for standard drug, 5 % (w/w) and 10% (w/w) extract ointment respectively. The 10% and 5% extract ointment reduce the period of epithelialization in a dose dependent manner (17.00 & 14.17 respectively). The 10% extract ointment treated group showed faster rate of epithelialization ($p<0.001$) compared to the negative control group. Moreover, 10% (w/w) extract ointment showed significant ($p<0.05$) difference of epithelialization period as compared to 5% (w/w) extract ointment treated group. However, the observable difference between epithelialization periods of the extract ointment (10% & 5%) treated groups and standard drug treated group was failed to reach statistical significance. Groups treated with simple ointment were failed to re-epithelise with in the follow-up periods (Table 8).

Table 8: Effect of topical application of the 80% methanolic extracts ointment of *A. polystachus on* the period of epithelialization of infected wound model in mice

Treatment groups	Period of epethelialization (days) Mean ± SEM
Simple ointment base	FR
Nitrofurazone (0.2% w/w)	16.17 ± 1.83[a3]
5% crude extract ointment (w/w)	17.00 ± 0.86[a3]
10% crude extract ointmetnt (w/w)	14.17 ± 1.17 [a3c1]

Note: FR= Failed to Re-epithelise; Values are expressed as mean ± S.E.M (n = 6); one way ANOVA. [a] compared to negative control(simple ointment), [c] compared to 5% ointment; [1]$P < 0.05$ & [3] $P < 0$ .001, when compared to control group; one way ANOVA.

4.5.3. Incision model

In incision wound model (Table 9), the standard drug, 10%(w/w) and 5%(w/w) extract treated groups showed significant increase in breaking strength (261.33±5.89, 267.50±7.61 and 258.50± 7.21 respectively), when compared to the negative control group(simple ointment) (197.00 ± 5.25). In this finding, the observable increase in tensile strength was found to be higher in 10% extract ointment as compared to nitrofurazone treated groups. However, this finding was failed to reach statistical significance. Similarly, there was no statistically significant difference between nitrofurazone and 5% (w/w) crude extract ointment treated groups. Moreover, there was no significant difference in breaking strength between 10% (w/w) and 5% (w/w) extract treated groups. The tensile strength was significantly increased by 35.79% ($p< 0.001$), 31.22% ($p< 0.001$) and 32.65% ($p<0.001$) for groups treated with 10% extract, 5% extract and nitrofurazone ointment treated groups, respectively (Table 9).

Table 9: Effect of topical application of the 80% methanolic crude extract ointment of *A. polystachus* leaves on tensile strength of incision wound model in mice

Treatment group	Tensile strength (g) (mean± SEM)	%Tensile strength
Simple ointment	197.00 ± 5.25	_
Nitrofurazone (0.2%)	261.33± 5.89[a3]	31.22%
5% crude extract ointment	258.50± 7.21[a3]	32.65%
10% crude extract ointment	267.50 ± 7.61[a3]	35.79%

Note: Values are expressed as mean ± S.E.M (n = 6); one way ANOVA. [a] compared to negative (simple ointment) control; [3] $P < 0$ .001, when compared to control group; one way ANOVA.

5. Discussion

5.1. Antibacterial activity

A. polystachus is one of the medicinal plants used for the treatment of wound, bacterial infections and other disorders. The present study was undertaken to determine whether the leaves of *A. polystachus* responsible for its antibacterial and wound healing activity. The result of the antibacterial activity test indicated that the crude extract was found to have greater antibacterial effect against *S. aureus* than the active solvent fractions. Whereas, the crude extract revealed almost similar antibacterial activity compared to ethyl acetate & aqueous fractions against *S. pyogen & P. aeruginosa*. However, the ethyl acetate and aqueous fractions showed similar antibacterial activity against *S .pyogens* and *P. aeruginosa* at 250 & 500mg/ml concentrations. Therefore, the enhanced antibacterial activity of the crude extract might be due to the synergistic or additive effect of the secondary metabolites that were relatively partitioned in the solvent fractionation which in turn caused the decrease in antibacterial activity observed in the solvent fractions. In spite of having antibacterial activities, the mean zone of inhibition of the crude and the active solvent fractions at all tested concentrations were not statistically comparable to that of their respective positive control for each of the susceptible bacterium. This might be due to the less concentration of the active principles in the crude extract & solvent fractions against the test bacteria.

The crude extract, ethyl acetate and aqueous fractions had similar antibacterial spectrum of activity against gram positive bacteria (*S. aureus & S. pyogens*) and gram negative (*P. aeruginosa*); whereas only the crude extract and chloroform fraction revealed antibacterial activity against *K .pneumonia*). However, *K. pneumonia* was insusceptible to ethyl acetate and aqueous fractions. This indicates that the active metabolites which inhibit the growth of these bacteria might be better extracted by 80% methanol (crude) and chloroform and may be immiscible in ethyl acetate and aqueous.

The difference also might be due to gram negative bacteria were less susceptible for the crude and the active solvent fractions of the study plant as they have an outer membrane. Therefore, the activity difference among the gram negative and gram positive bacteria could be because of the partial penetration of the bioactive metabolites through the lipopolysaccharide rich outer cell membrane in the cell wall of gram negative bacteria unlike in the cell wall of the gram positive bacteria with less effective permeability barrier (91). This reason is further strengthened by the differences in the composition and concentrations of secondary metabolites in the ethyl acetate (absence of glycosides) and aqueous fractions (devoid of terpenoides & glycosides). Moreover, the differences in the antibacterial spectrum of the ethyl acetate, aqueous and the chloroform fractions against the test bacteria might be linked to the partitioning or localization of secondary metabolites in the respective fractions as the plant had been extracted in sequential solvents of increasing polarity.

For instance, unlike the ethyl acetate and aqueous fractions, the presence of glycosides in the chloroform fraction could directly or indirectly enhance its bacterial growth inhibitory effects on gram negative bacteria such as *K. pneumonia* (92).

In the present study, *E. coli* was found to be insusceptible against the crude extract and solvent fractions at all tested concentrations. The possible reason for the less susceptible nature of these bacterial species could be due to the presence of outer cell membrane permeability barrier in gram negative bacteria, so that the bioactive compounds may be failed to penetrate across this barrier. In addition, the induction of efflux pump or biofilm formation could hinder the antibacterial activity of the bioactive compounds (93).

The lack of antibacterial activity in the chloroform fraction might be supported by the fact that most of the secondary metabolites of the extract may be polar compounds and hence could be extracted by the more polar solvents used sufficiently rather than being extracted by less polar one such as chloroform(94). In addition, the absence of antibacterial activity of the chloroform fraction against *S. aures, S. pyogen & P. aeruginosa* might be further strengthened and associated with the absence of the secondary metabolites tested such as tannins and flavonoids, as displayed in the preliminary phytochemical screening test (Table 2). This result is supported by similar studies conducted on Acantheceae family. The ethanolic and ethyl acetate fractions of the roots of *Acanthus pubescens* revealed similar antibacterial activity against *P. aurugenosa, S. aureus & Streptococcus species* (70). Likewise, the ethanol, methanol and aqueous extracts of the different parts of the *Acanthus ilicifolius* exhibited strong to moderate activity against gram positive and negative bacteria (95). Furthermore, antimicrobial activity test of the leaf extract of acanthus montanus showed moderate to highest antibacterial activities. These observed differences can be attributed to distribution of active principles (tannins, glycosides, polyphenols, terpenes, flavonoids) in different fractions due to their affinity with the solvent used during fractionation (96).

The result of antibacterial susceptibility test (in terms of zone of inhibition) of the crude and the active solvent fractions against the respective susceptible bacteria were inversely proportional to their MIC and MBC values. This implies that the more susceptible the bacteria to the crude extract or the solvent fraction, the less is its corresponding MIC and MBC values, suggesting the reproducibility and consistency of the experiments. In addition, in almost all findings, the MBC value was one or two dilution factor greater than that of the MIC value for each individual bacterium in the micro-broth dilution tests of crude extract and the active solvent fractions; this might indicate the sensitivity of the dilution method in detecting the minimum bacterial turbidity (growth of bacteria) (97).

Phytochemicals such as polyphenols, terpenoids, anthraquinones and saponnins were confirmed in the crude extract & all the solvent fractions.

However, tannins & flavonoids were detected only in the ethyl acetate and aqueous fractions; whereas, glycosides were detected in the crude extract and chloroform fraction. Therefore, the presence or absence of these phytochemicals in the crude extract and solvent fractions may contribute the difference in antibacterial activities of the leaf extracts of *A. polystachus.*

The above reasons can be evidenced by different studies conducted on phytochemical compounds such as flavonoids, alkaloids, tannins, tepenoids, essential oils, saponins, glycosides and phenols found to have antibacterial activities (10). Furthermore, study on *Acanthus ilicifolius* (Acanthaceae family) indicated that flavonoids and terpenes are responsible for the antimicrobial activity of ethanol, methanol and aqueous extracts against gram positive and negative bacteria (95).

Even though, it is difficult to judge the mechanism of actions of the bioactivity of the crude and the bioactive solvent fractions of the study plant, it is plausible to speculate their antibacterial effect based on the different mode of action of the bioactive phytochemicals confirmed in different studies.

To mention some of the phytochemicals having antibacterial activities with their possible mechanisms; flavonoids and their derivatives or congeners like quercetin, naringenin(98), apigenin, luteolin, crycristagallin, and orientanol B have been found to have antimicrobial activities via damage or disruption of the cell membranes and inhibition of the synthesis of nucleic acids which can lead to the death of the susceptible bacterium (99). Phenolic compounds (isolated from *Carum carvi*) and (oolong tea) found to have a growth inhibition effect against susceptible bacteria via disrupting the metabolic function (forming heavy soluble complexes with enzymes) and with their possible attack on the cell walls of bacteria (100, 101).

Additionally, tannins isolated from green tea leaf & its derivatives (catechin, ellagetannin and gallotannin) found to be active against the growth of susceptible bacteria by damaging the cell membrane and inhibition of metabolic pathways like oxidative phosphorylation (102, 103). Terpenoids isolated from *Luffa cylindrical* (83), anthraquinones such as emodin (isolated from *Rheum officinale)* (104) and 1, 8-dihydroxy-anthraquinone (isolated from *Porphyra haitanensis*) (105), saponnin isolated from *sorghum bicolor* (106) and *Acacia aroma* (107) were found to have antibacterial activities against gram positive and negative bacteria by the disruption of cytoplasmic cell membarane, interaction with the bacterial cell wall and cell membrane which can lead to the death of the bacteria as a result of leakage of cytoplasmic components and loss of cell integrity (83, 104-108). Therefore, the presence of flavonoids, tannins, polyphenols, terpenoids, saponinns and glycosides may contribute the antibacterial activities of the extracts.

Generally, antibacterial activity screening results are still indicative of the potential of the leaves of *A. polystachus* as effective medicaments in the treatment of infections caused by the tested bacteria. This effect might be attributed to either the individual class of compounds present in the crude and the active solvent fractions may conceivably cause additive or synergistic effect that each class of compound exerted to give the observed antibacterial activities.

5.2. Wound healing activity

Wound healing is a sequence of events which consists of coagulation, inflammation, collagenation, wound contraction and epithelialization. While the phase between coagulation to collagenation is intimately inter-linked, the phase of wound contraction and epithelialization are independent to each other and run concurrently(109). Drugs, which influence one phase, may not necessarily influence another. Thus, the development of an ideal wound healing drug is still a challenge to the medical scientists. The ideal drug should fulfill the criteria such as rapid contraction of wound leading to quick healing, reduction of wound epithelialization periods and appreciable gain of tensile strength(109).

Because the use of a single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing, two or more models are used in wound healing studies. Even though a large effort has been made to study *in vitro* wound healing activity, *in vivo* studies are still remain indispensable for wound healing activity investigation as wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. In order to evaluate wound healing activity *in vitro* study and single model are not adequate to collectively represent the various components of the wound healing process as a whole (110). Hence, in the present study three different models were used to establish the *in vivo* wound healing potential of 80% methanolic crude extracts of *A. polystachus* Delile leaves on various phases.

Traditionally, the leaves of *A. polystachus* are used for wound healing activity mixed with butter. Applying the extract directly on the affected wound can't bring the desired effect as they don't stay longer on the wounded skin of the experimental animals. Semi-solid preparation like ointment is necessary to achieve a sustained drug release at the application sites. Hence, a hydrophobic base (simple ointment) was selected based on the leaves of *A. polystachus* traditional wound healing use having assumption that a polar extract would be released better from the non polar base and vice versa(52). The ointment base has multiple roles; the hydrocarbon bases such as hard paraffin and white soft paraffin are used to form an occlusive barrier on the skin that prevents escape of moisture from the skin into the environment. As a result, moisture accumulates between skin and ointment layer that causes hydration of the stratum corneum.

Hence, the moisture layer provides a medium for dissolution of the drug that is otherwise dispersed as fine particles in the ointment base. Wool fat and Cetostearyl alcohol are thickeners and they are used for stabilization of ointment(80).

The results of this study on wound healing activity revealed that the crude extract significantly promote wound healing effects with both 10%(w/w) and 5%(w/w) extract ointment treated groups in the excision, infected and incision wound models as compared to negative control group. This can be supported by the fact that the greater the reduction in the rate of wound contraction, the better is the efficacy of medication and the wound will close at fast rate if the medication is more efficient (111).

In excision wound healing model, the crude extract (80% methanol) of the leaves of *A. polystachus* showed statistically significant wound area contraction compared to the negative control. The 10% (w/w) extract ointment treated group revealed faster wound area contraction from day 6 to day 16. Whereas, the 5% (w/w) extract ointment treated group showed statistically significant wound area contraction starting from the 8^{th} day onwards. Although the observed effect was better in 10% (w/w) extract treated group in contrast to the 5% extract & standard drug (nitrofurazone) treated groups, this difference was failed to reach statistical significance. The higher wound contraction rate of the extract ointment may be due either to its higher antibacterial effect or induction of macrophage cell proliferation. The faster rate of contraction with the standard drug may be attributed to its antimicrobial effect (112). Furthermore, the period of epithelialization was significantly reduced from 20 days (negative control) to 15, 15 and 13 days for 5% extract, nitrofurazone and 10% extract ointment treated groups, respectively. The shorter period of epithelialization & faster wound area contraction could be due to the ability of *A. polystachus* leaf extract to enhance collagen synthesis, induction of cell proliferation & antimicrobial activities of bioactive constituents(43).

In the case of infected wound model, the ointments of crude extract revealed statistically significant wound healing effect in mice infected with *S. aureus*. The infiltration, blister formation, edema and exudates exhibited on the wounds of mice before treatment were vanished in all treated groups except the negative control. Groups treated with 10% extract ointment showed faster rate of wound contraction than nitrofurazone and 5% extract ointment treated groups. Additionally, the period of epithelialization was shorter in 10% extract ointment (14.17) followed by nitrofurazone (16.17) and longer in case of 5% extracts ointment (17.00) treated groups as compared to negative control. This finding indicated that the wound healing activity of the extract in infected wound model presumed to be dose dependent manner. In this study, the antibacterial activity of the extract was confirmed against common wound infecting pathogens which might contribute remarkably to the faster wound healing rate. Supporting evidence explained that the eradication of the colonizing organisms from infected wounds creates a suitable environment for wound healing to take place(24, 48).

Moreover, groups treated with simple ointment exhibited slow rate in wound area contraction & failed to re-epithelialise with in the follow-up periods. This is perhaps infection stuck wound healing even lead to death. This is because one of the major obstacles in wound healing process is infection which is known to interrupt healing and at worst can cause death.
In chronic wounds (such as infected wound), it has been suggested that bacteria & fungi delay healing. As a result, the antimicrobial activity could be a key factor in the rapid wound healing process & much attention has been directed toward extracts of the plants with antimicrobial activity in treatment of infected wounds (24, 48).

In incision wound model, significant increase in skin breaking strength was observed. Groups treated with 10% and 5% (w/w) extract & standard ointments showed statistically significant increase in tensile strength as compared to simple ointment base treated group. However, the difference in tensile strength was not statistically significant among standard drug, 10% & 5% (w/w) ointment treated groups. The increase in tensile strength in the incision model may be due to the antioxidant activity of the extract, increase in collagen synthesis & maturation, formation of stable intra- and intermolecular crosslink, matrix deposition and cell migration. This evidence is supported by the fact that increase in skin breaking strength in incision model indicated enhanced collagen maturation. Since, collagen is the major protein of extracellular matrix which gives strength, support and integrity to the wound matrix (113). The healing process depends to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation (43). Any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, by increasing the circulation, by preventing the cell damage and by promoting the DNA synthesis (111). Thus, the presence of more than one compound in a plant extract could contribute to the observed net pharmacological response (114).

The first probable justification for the wound healing activity of the plant is the crude extract might enhance collagen synthesis perhaps accelerates wound healing process. This is supported by other studies which showed that wound contraction begins almost concurrently with collagen synthesis and re-arrangement of collagen fibers through the action of fibroblasts and increases the viability of epithelial cells (115). For instance, iridoid glycosides isolated from the *Acanthaceae* family and flavonoids are known in promoting wound healing (67, 111, 113).

Another possible rationale for the wound healing effect of the crude extract is that the extract might promote induction of macrophage cell proliferation & facilitate proliferation of epithelial cells thereby hasten wound contraction by enhanced epithelial migration.

The effect of macrophage cell proliferation in wound healing was evidenced by the fact that, after injury macrophages enter the site and support the ongoing process by performing phagocytosis of pathogens and cell debris, secreting growth factors, chemokines and cytokines, promotion and resolution of inflammation, the removal of apoptotic cells and the support of cell proliferation and tissue restoration. Besides, macrophages evidently play an integral role in a successful healing response through the synthesis of potent transforming growth factor (TGF-β, TGF-α, platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which promote cell proliferation. Macrophages also mediate angiogenesis, synthesize nitric oxide and form fibrous tissue. Thus, they are essential for the transition from the inflammatory to the repair phase because of their essential role in wound healing process (28).

In addition, studies have shown that antimicrobial activity of various plants support wound healing(116). Promoting wound healing in animal groups treated with crude extract of leaves of *A. polystachus* may also be due to the antimicrobial activity of bioactive constituents. This is further strengthened by the fact that in this study, *in vitro* antibacterial activity of the crude extract and solvent fractions of *A. polystachus* leaves was tested and found to have antibacterial activity against common wound pathogens such as *S. aureus, P. aeuruginosa* and *S. pyogens* which could support the wound healing activities. Moreover, studies revealed that medicinal plants such as *Dissotis theifolia* (24) & *Piper hayneanum* (48) which have antibacterial & antifungal activities also possess wound healing effects. The effect of antimicrobial activity support wound healing; because, infection can seriously delay healing process by disrupting the normal clotting mechanisms, promoting disordered leukocyte function and ultimately delaying angiogenesis, cause poor quality granulation tissue formation, reduced tensile strength of connective tissue, impaired epithelization (116). Both microbes and endotoxins can lead to a prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. This can cause a chronic inflammatory state that promotes the development of Matrix metallo proteinases (MMPs), thus inhibiting wound healing. Bioactive metabolites, such as flavonoids, tannins, glycosides and terpenoids promote the wound-healing process mainly due to their astringent and antimicrobial property (117).

Another possible reason for enhanced wound healing effect could be due to the crude extracts of *A. polystachus* leaves which may possess antioxidant, scavenging free radicals and cell proliferating properties. Evidence suggests that antioxidants may be of therapeutic use in wound healing by promoting wound contraction & epithelialization (31, 38). This is because; the presence of free radicals will result in oxidative stress leading to lipid peroxidation (LPO), DNA breakage, and enzyme inactivation (free-radical scavenger enzymes). The increase in free radical production and diminished antioxidant activity may worsen the condition and account for the delay in healing.

While they fight pathogens, neutrophils release inflammatory cytokines, ROS and enzymes that damage cells and prevent cell proliferation and wound closure by damaging DNA, lipids, proteins, enzyme inactivation (free-radical scavenge), the ECM and cytokines that speed healing. Hence, agents that demonstrate significant antioxidant & scavenging free radicals activity may, therefore, preserve viable tissue and facilitate wound healing(31, 35).

The role of antioxidant and free radical scavenging property in wound healing process is further strengthened by other studies conducted on the *acantheceae* family which revealed that the plant possess antibacterial, antifungal, anti-inflammatory, antipyretic, antioxidant, antiviral and immunomodulatory effects (67). To mention some, a study on the leaf and root extracts of *Acanthus ilicifolius* showed antioxidant activity & scavenging free radicals (superoxide and hydroxyl radicals, inhibit the generation of lipid peroxides radicals) due to the presence of flavonoids (95). In addition, the root extract of *Acanthus sennii* revealed that the presence of glycosides, flavonoids & polyphenols. Especially, Iridoid glycosides isolated and validated in the *Acanthaceae* family possess antioxidant, antimicrobial, analgesic, antitumor and anti-inflammatory properties (67, 118).

Furthermore, the anti inflammatory activity perhaps contributed for the wound healing effects. The normal function of inflammation in an acute wound is to prepare the wound bed for healing by removing necrotic tissue, debris, and bacterial contaminants as well as recruiting and activating fibroblasts. However, excessive inflammation is a major contributing factor to the persistence of chronic non-healing wounds, which are “trapped” in the inflammatory phase of wound healing and fail to re-epithelialise (119). Even though many anti-inflammatory agents were used for wound treatment, they are well known to inhibit wound repair via comprehensive anti-inflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis (31, 120). The polyphenols, glycosides & flavonoides present in this crude extract may be responsible for the anti-inflammatory activities as evidenced by different studies (120). For instance, compounds such as polyphenols, glycosides & flavonoids (prevent the synthesis of prostaglandins) possess anti-inflammatory activities (121). Glycosides (Iridoid glycosides) isolated from the same family (*acantheceae*) possess anti-inflammatory and analgesic effects (118). Furthermore, previous reports indicated that a number of plants with anti-inflammatory activity do also possess wound healing effects. These include Centaurea iberica Trev, *Ficus religiosa,* Curcuma aromatica and Memecylon edule Roxb (122). Hence, phytochemical constituents of *A. polystachus* leaf crude extract seem to contribute to its wound healing activity either by additive or synergistic effect of these secondary metabolites.

The role of phytochemicals in wound healing is also supported by different studies. For instance, tannins are seen to be active detoxifying agents and inhibit bacterial growth(123); terpenoids promote the wound-healing process mainly due to their astringent and antimicrobial property (117); flavonoids are potent antioxidants, free radical scavengers (111, 113). Polyphenols & flavonoids (prevent the synthesis of prostaglandins) possess anti-inflammatory and have antimicrobial activities (121). Glycosides (Iridoid glycosides) isolated from the same family (*acantheceae*) possess antioxidant, antimicrobial, analgesic, antitumor, immunomodulatory and anti-inflammatory effects (118). Therefore, the presence of phytochemicals in the crude extract (such as terpenoids, flavonoids, glycosides, saponins, tannins and phenolic compounds) may contribute in the antibacterial and wound healing activities either independently or additive/synergistic effects.

6. Conclusion and Recommendation

6.1. Conclusion

The present study revealed that the crude extract (80% methanol), chloroform, ethyl acetate and the aqueous fractions of the leaves of *A. polystachus* possess antibacterial activities against the growth of selected pathogenic bacteria with varying antibacterial spectrum. Therefore, the study provides scientific basis on the traditional claimed use of the medicinal plant for the treatment of bacterial infections like syphilis, trachoma, gonorrhea or others which are probably caused by the susceptible bacteria.

In this study, in all the three models the different phases of wound repair, such as wound contraction, epithelialization and tensile strength were enhanced by the 80% methanolic crude extract ointment of the leaves of *A. polystachus* as compared to the negative control group. These results collectively demonstrate that the 80% methanolic extract possesses wound healing activity and this justifies the use of the leaves of *A. polystachus* for treatment wounds as claimed in the folklore literature. This study also showed that the crude extract of *A. polystachus* was endowed with significant antibacterial activities that explain at least in part its wound healing activity.

6.2. Recommendations

- The results of the present study should be corroborated with histopathological studies
- As chronic wounds such as diabetic wounds are major global burden, it is worthwhile to study the activity of the plant on chronic wounds
- Further studies should be conducted to isolate, purify and identify bioactive principle(s) responsible for the antibacterial and wound healing activities of the plant.
- Mechanistic studies for agents responsible for antibacterial and wound healing activities of the study plant have to be conducted.
- The chronic toxicological studies should be done to confirm the safety of the extracts of the plant on chronic uses.
- At last, the antibacterial activities of the plant should also be done on other bacterial species which were not addressed by this study.

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

Name: Wubante Demilew
Signature: _____

This thesis has been submitted for examination with our approval as University Advisors.

Name of Advisor	Signature	Date
1. Mr. Seyfe Asrade	_____	_____
2. Mr. Getnet Mequanint	_____	_____

Place and Date of Submission: Gondar, Ethiopia, June, 2017